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INTERNATIONAL WEEKLY JOURNAL OF SCIENCE

Vol 312 No 5989 1-7 November 1984 £1.80 \$4.50

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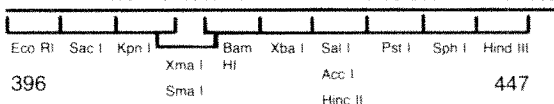
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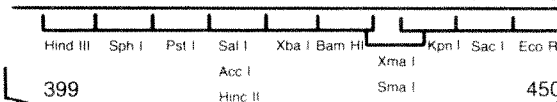
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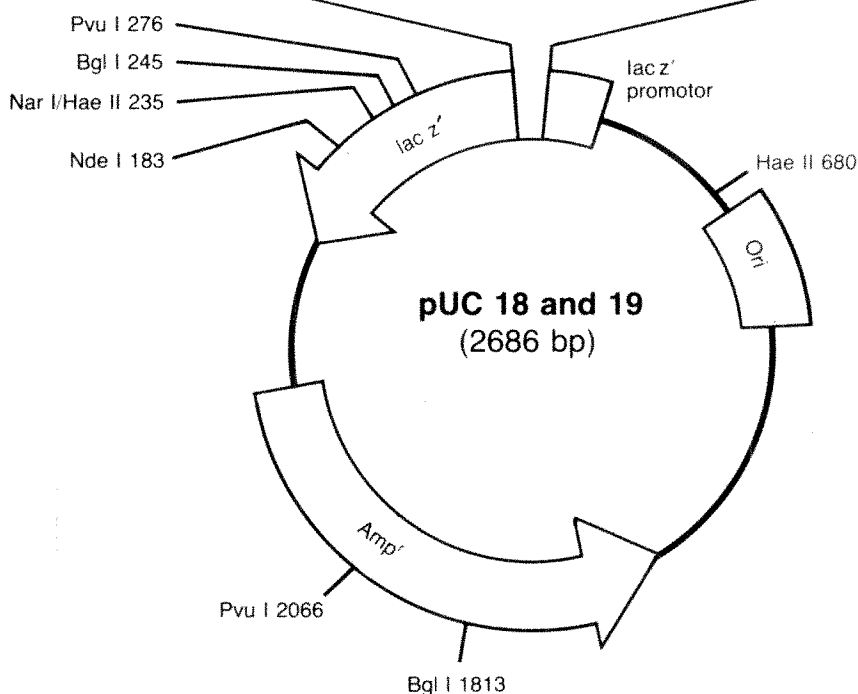


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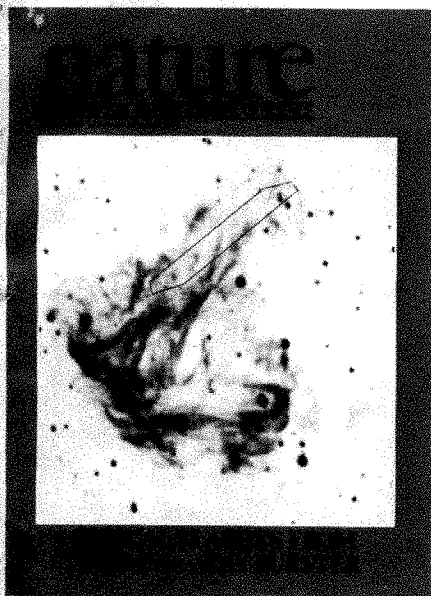
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The N49 supernova remnant in the Large Magellanic Cloud, which may contain the γ -ray burst source GBS0526-66 (in error box shown above). The picture results from a 20-min exposure in visual light, obtained with a CCD camera at the 2.2 metre telescope at the ESO/La Silla observatory in Chile. (See p.46.)

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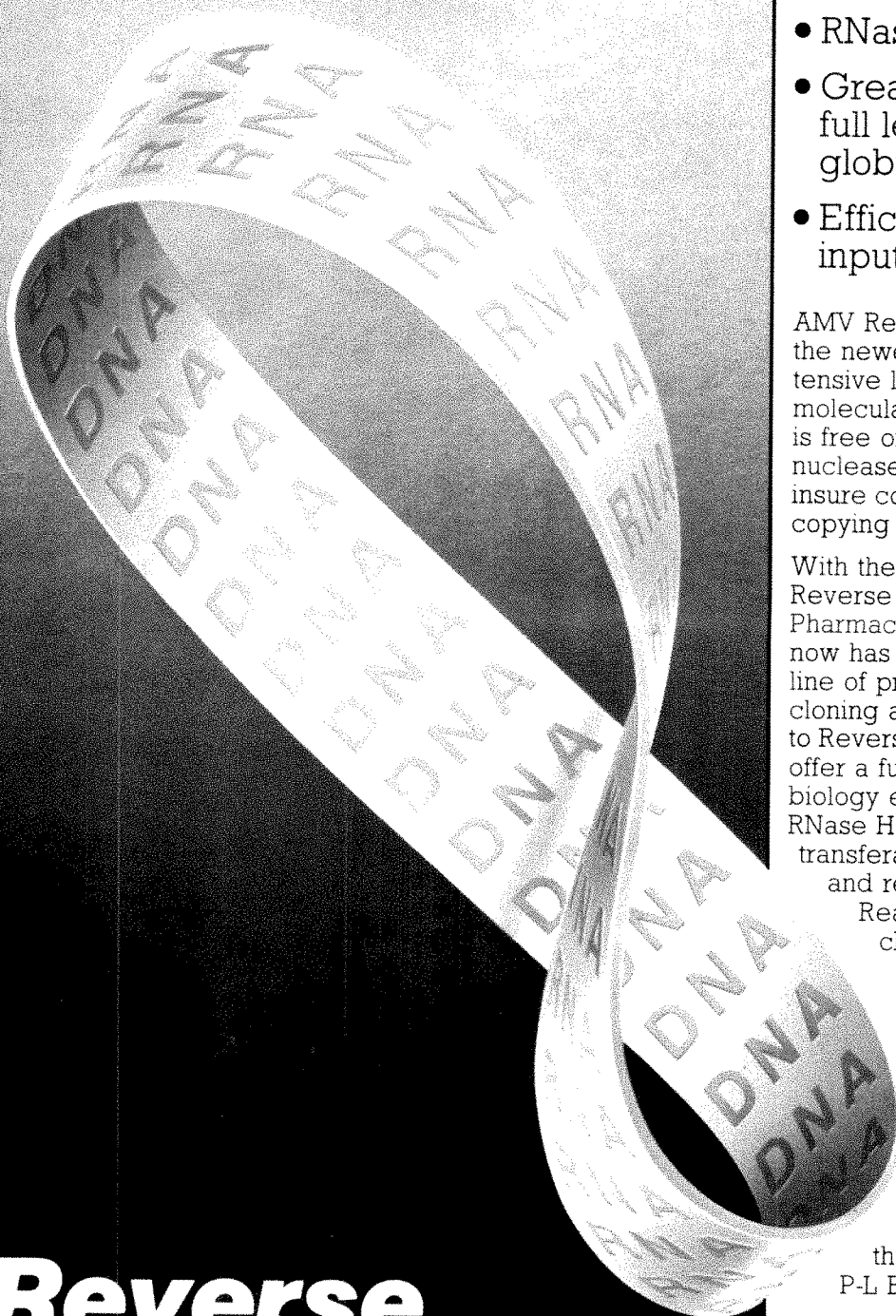
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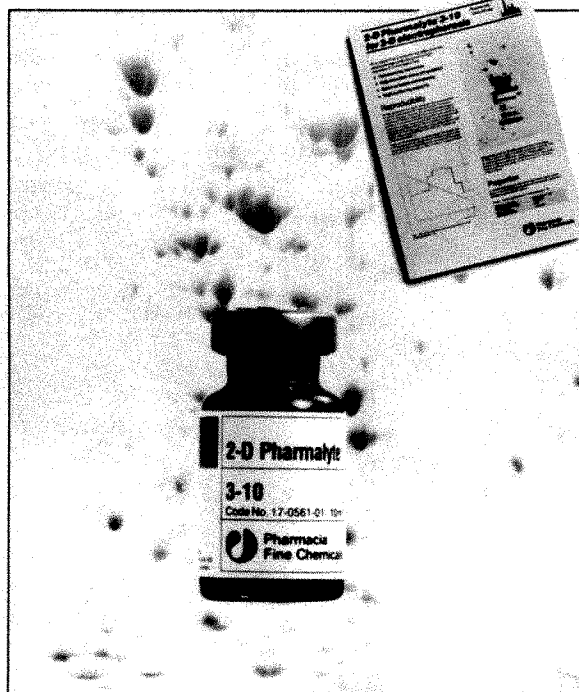
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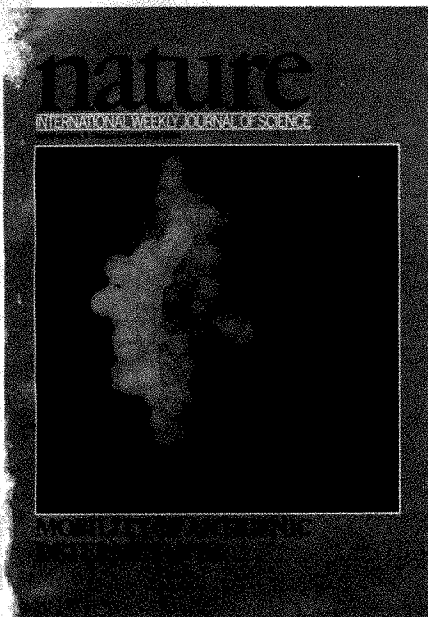
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COVER



A raster graphics illustration of the pattern of temperature factors (a measure of mobility) on the molecular surface of myohaemerythrin. Dark colours represent the lowest and bright colours the highest factors. The method has been used to study how antibodies interact with different regions of a protein molecule. (See page 127.)

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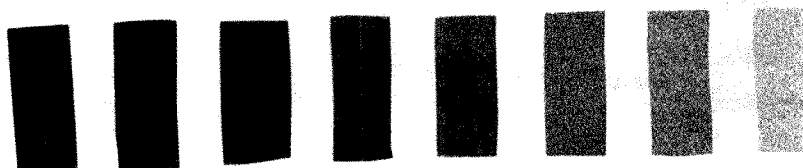
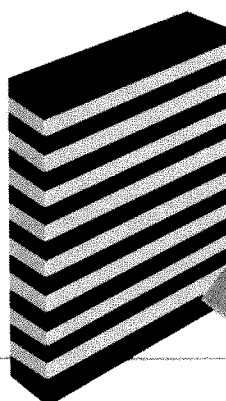
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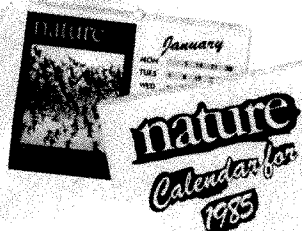
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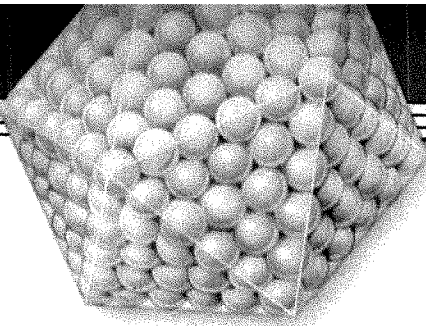
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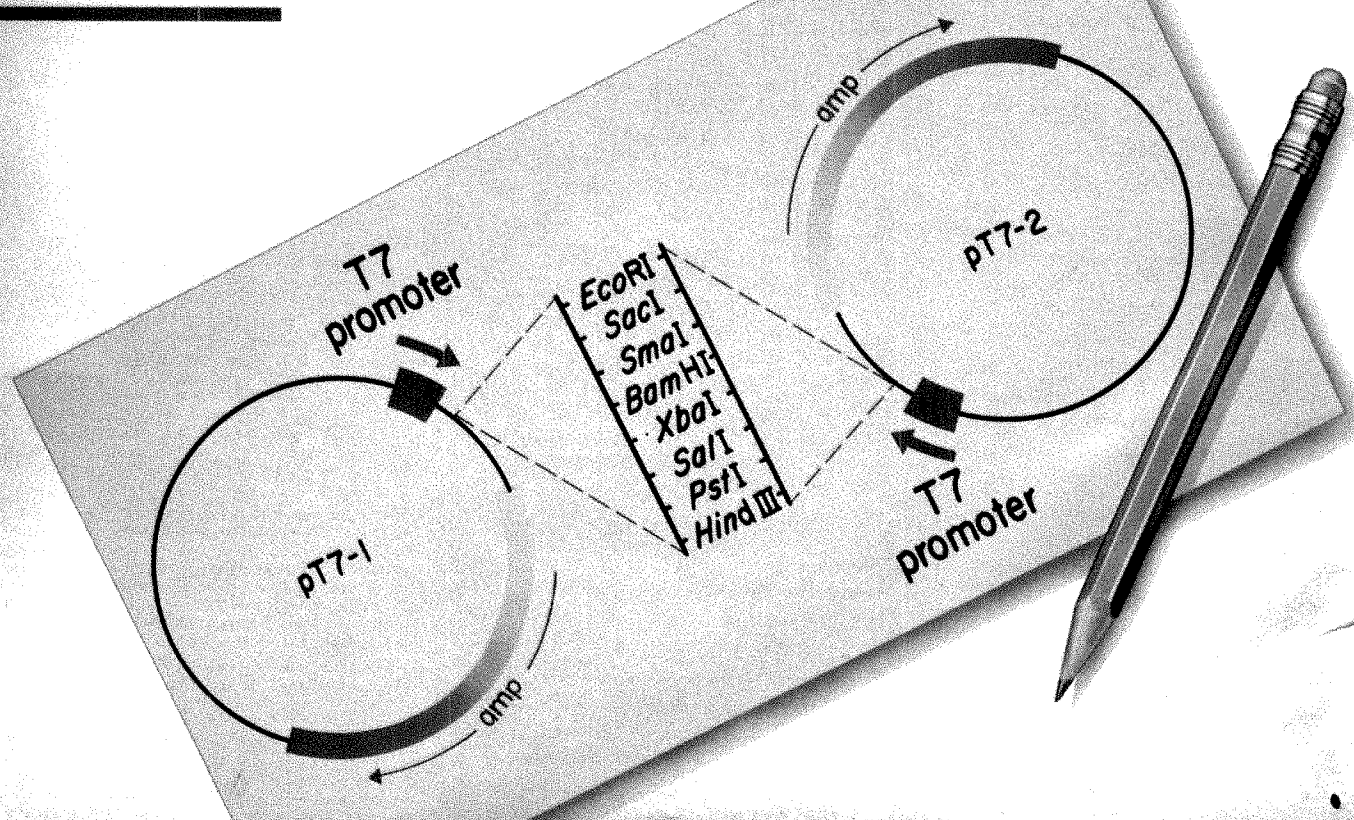
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A fluorescence micrograph of closed and open stomata from *Commelina communis* stained with chlortetracycline. Nuclei are seen as yellow fluorescence and chloroplasts red. See page 361. (Photo: W Papal & R Hedrich.)

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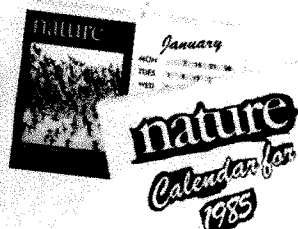
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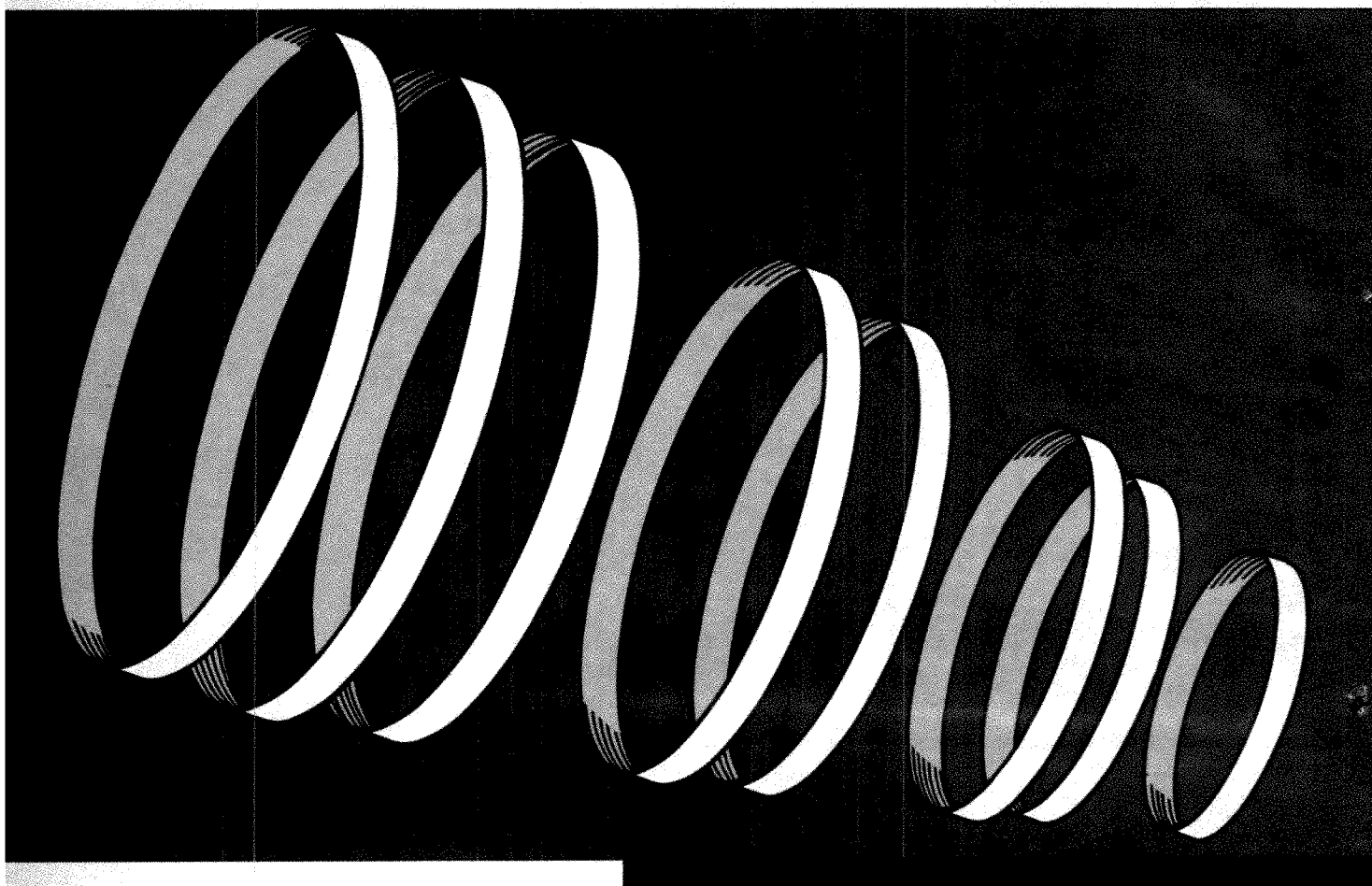
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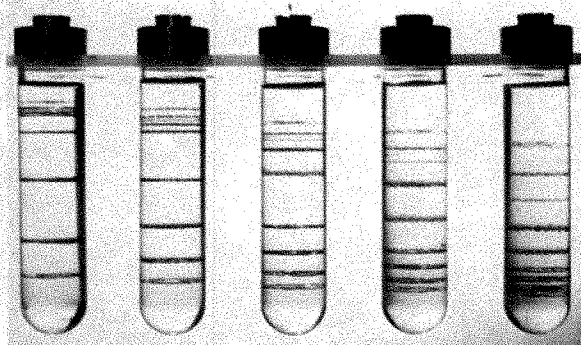
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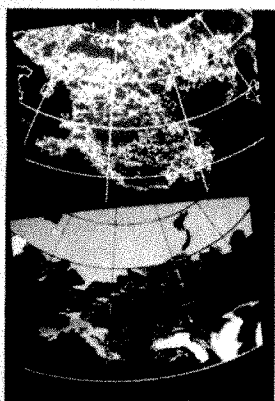
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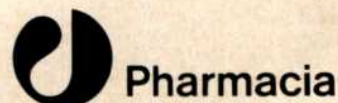
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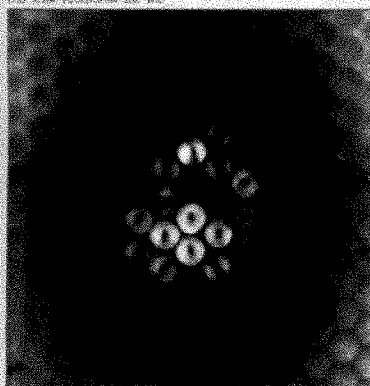
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STANDING WAVES IN
A COMPOUND EYE

The long narrow photoreceptors in many insect compound eyes behave like waveguides, and light propagates along the receptor in the form of 'modes' or standing waves. In butterflies (here *Heteronympha erope*) these modes are imaged onto the corneal surface by a powerful lens found close to the tip of the photoreceptor (see page 561). (Photo: D.-E. Nilsson.)

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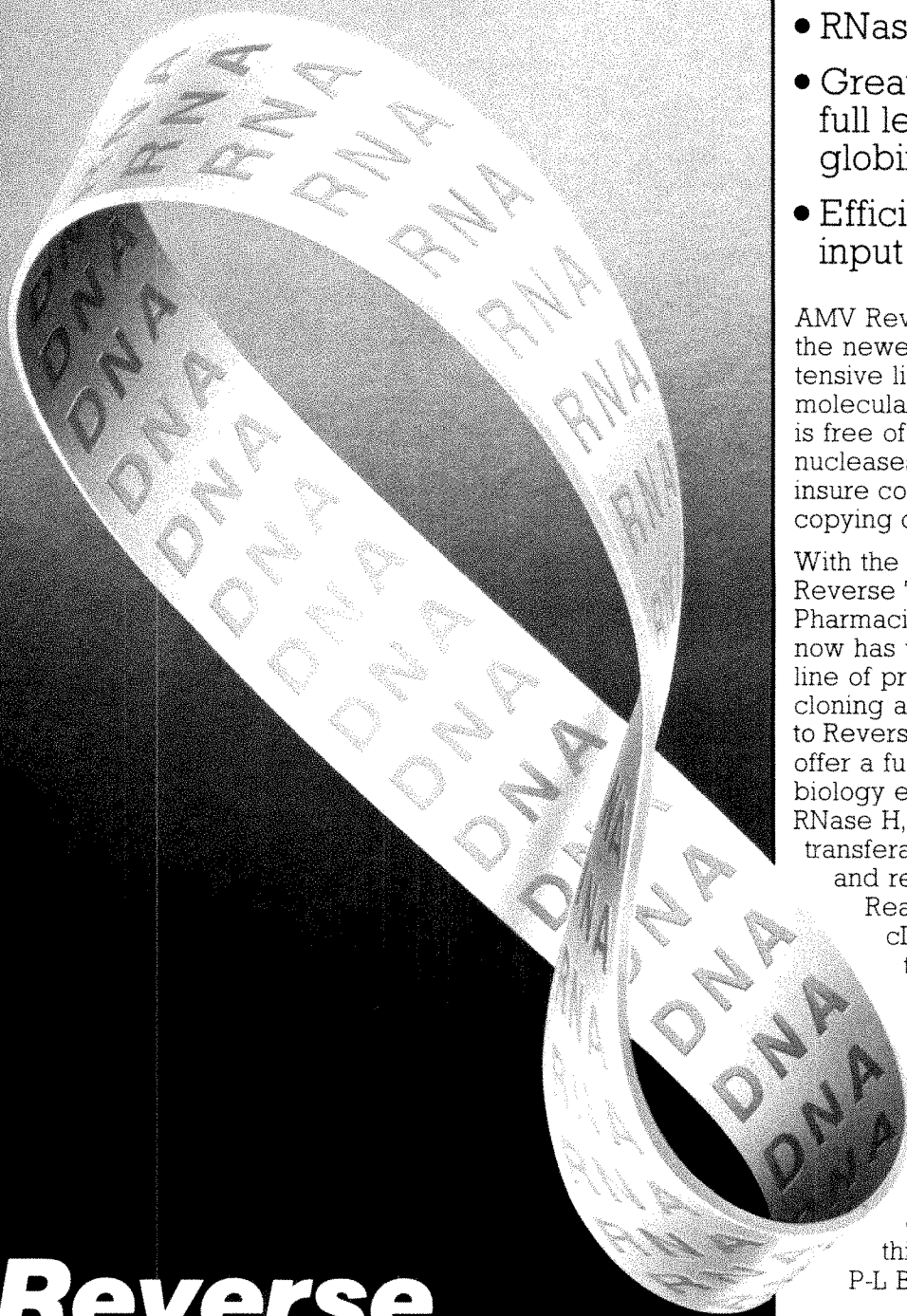
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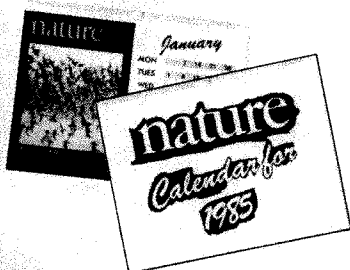
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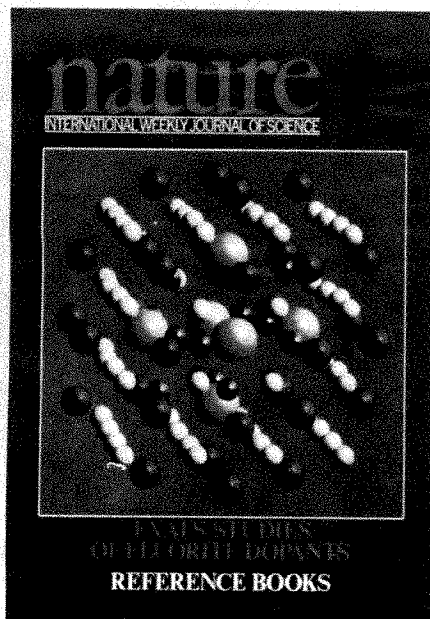
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A representation of the hexameric rare earth dopant cluster in CaF_2 showing the minimum energy configuration after relaxation of the structure by computer simulation. Yellow spheres represent the six trivalent rare earth ions; red spheres, the interstitial, charge-compensating fluoride ions, and blue and white spheres, the lattice calcium and fluoride ions, respectively. See page 601.

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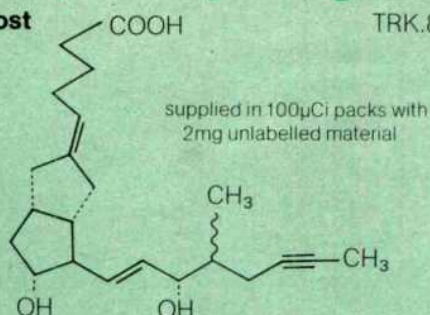
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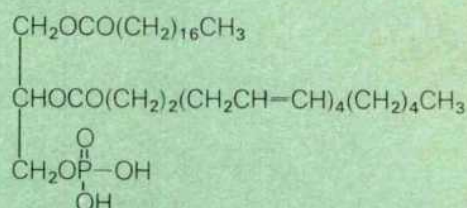
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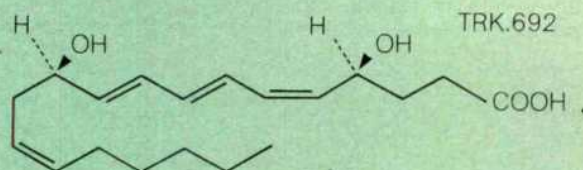
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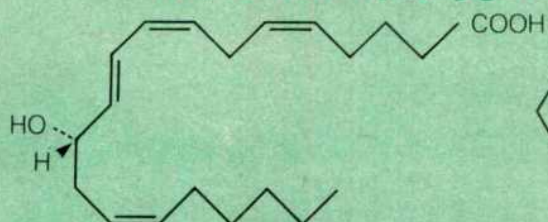
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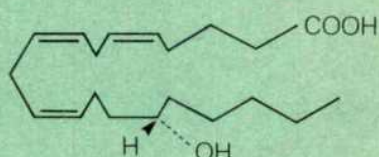


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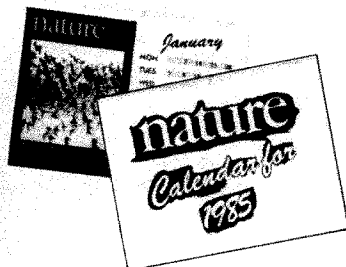
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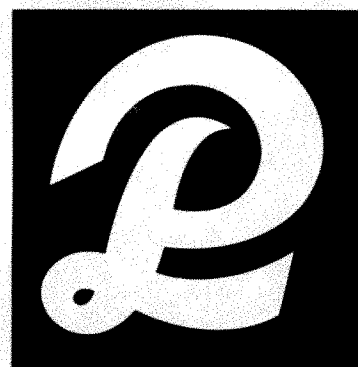
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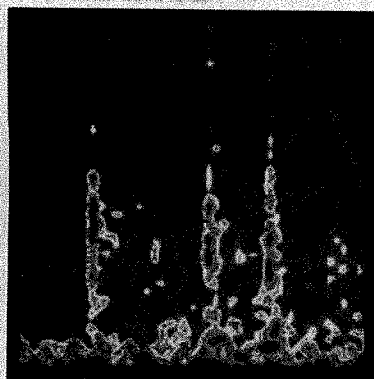


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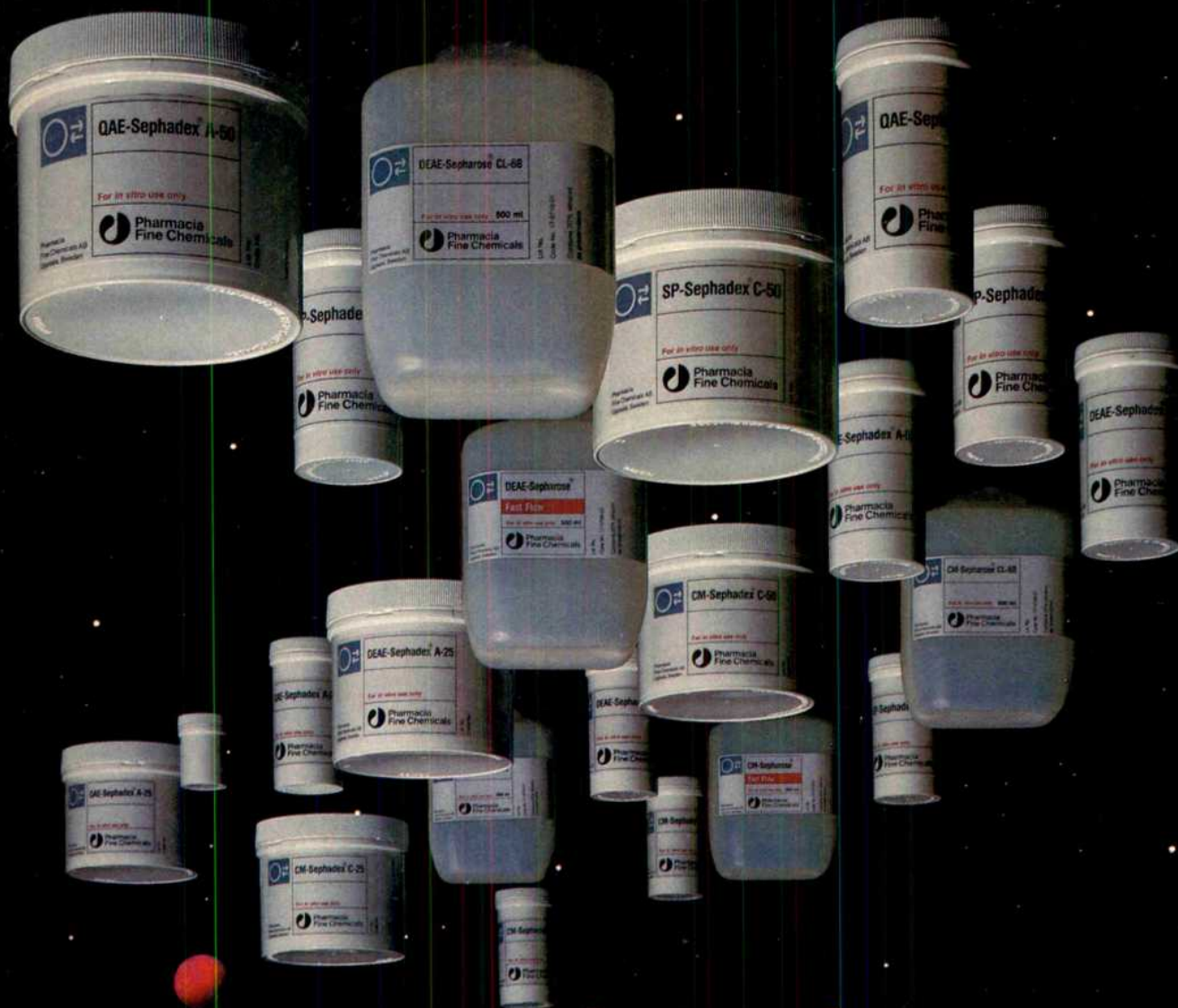
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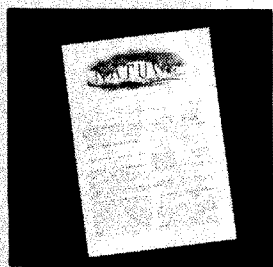
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Nature® ISSN 0028-0836

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Vol.312 No. 5996 20/27 December 1984

Another monopoly on the block

This month's sale of the telephone company British Telecom will not bring competition but may net the government £4,000 million. The objectives are admirable but the methods are wrong.

JUST as there is no general answer to the question how long is a piece of string, so there is no easy way of telling what a national telephone network may be worth. That may be the most charitable excuse for the stratagems to which the British Government has gone, in the past few months, for selling off 51 per cent of the shares in its nationalized telecommunications industry, now trendily called British Telecom. Last week, after an advertising campaign intended to interest men in the street in becoming shareholders (and which must have done more for general business education than decades of formal education), British Telecom and its merchant bank last week published the draft of the prospectus on which its shares will be sold on 28 November. The only missing ingredients in the prospectus are the price at which shares will be offered for sale, and the precise proportions that will be set aside for employees of the company, investors overseas and so on. Everybody is a little agog that on this one day, anything up to £4,000 million may change hands (although only 40 per cent of it will be payable on the nail).

While there is every reason to applaud the government's chief objective, that of introducing some of the self-balancing discipline of the marketplace into the regulation of a gigantic industry, suspicion must persist about its secondary objectives (balancing its budget by selling off its capital assets) and there are grave doubts about its methods.

The laudable primary objective is now quite widely shared. Only this year, the US telephone network, AT&T, has been broken up into a number of smaller units, with geographically restricted monopoly rights. The Japanese Government is dicker with similar schemes. Everywhere, monopolistic telecommunications have become technically backward (or less technically advanced than they should be) and commercially slothful. Two quite separate tendencies have made the telephone monopolies indefensible. First, as experience in the United States has shown, allowing small companies to offer "value added services" by renting trunk circuits from the monopoly and selling on their use can benefit both the entrepreneurs and their customers. (The monopolies protest that these parasitic middlemen are merely skimming the cream off their legitimate business, to which the answer is that they should design their tariffs more intelligently.) Second, technology has often left the monopolies standing almost still and, in the past decade, with satellite communications in particular, has begun to raise the question whether telephone systems need now be the monopolies that seemed inevitable in the days of Alexander Bell. The British Government deserves some praise for recognizing that the time has come for change.

The British solution to the problem of the monopoly is at best half-hearted. There are three innovations. First, half of the telecommunications will in future be owned by private investors, not the government. The chief practical consequence will be that British Telecom will in future be able to raise capital from private sources without embarrassing the Treasury by inflating what is called the Public Sector Borrowing Requirement (which will assist technical innovation). Especially because the government promised, in last week's prospectus, not to vote its shares in ordinary circumstances, this change by itself is not a safeguard for the users of the network but, if anything, a device whereby the new owners can decide what profit they should be making. To guard

against exploitation, there are two safeguards — a regulatory office (called Oftel) with powers to rule on complaints of unfair competition which has yet to show its teeth (but they look sharp) and a requirement imposed by the government that domestic telephone tariffs should not in the next five years increase by more than two per cent less than the inflation rate (which is meaningless, because the new corporation is almost certain to wish to finance a greater share of its development by borrowing than in the past). Third, there is a simulacrum of independent competition, a quite separate telecommunications network called Mercury.

The best that can be hoped for, in these novel circumstances, is that the competition will prosper. The worst possible outcome is unthinkable, for the political trouble there would be if Mercury should be in danger of collapse would have the government and the Bank of England at its side in a flash. (Perhaps the moral of last week's prospectus is to invest in Mercury, not British Telecom.) Meanwhile, the British Telecom prospectus cheekily reminds potential investors that the restriction of its tariffs laid down by the government will apply to only 45 per cent of its business. (To be fair, the rest includes international telephone traffic, where competition is likely in due course to be fierce, and the sale of equipment, where it is already cutthroat.)

Meanwhile, the question remains how much the business may be worth. The government's embarrassment is that there is no objective way of putting a value to its worth. The value of the income stream from a monopoly enterprise is not necessarily related to anything but the decisions taken by individuals as to what the profit should be. The value of the network's assets, calculated from the cost of providing them in the first place but abated by depreciation, may conceal as assets equipment so inefficient that, in a competitive world, it would be thrown away. One of the curious anomalies in last week's prospectus is the brief statement on depreciation policy, disclosing that British Telecom reckons on an average life of between 6 and 20 years for its old Strowger electromechanical equipment, but a uniform ten years for new digital equipment.

The sale now arranged has inevitably an air of unreality. Ultimately, what will matter is how much people are prepared to pay for half of British Telecom. The best way to have found out would have been to put the shares on sale in dribs and drabs, on exactly the pattern in which the government itself sells government stock. The way in which things have been arranged, however, is certain to cause embarrassment. If the shares on offer are not taken up, the government will be laughed at. If on the other hand, the shares sell like hot cakes, it will be accused of having given away a public asset. It has only itself to blame. □

Research competition

British universities had better become knowledgeable about their research.

THOSE who think that the long agony of the British academic research enterprise must be coming to an end had better think again. That is the simple lesson to be drawn from last week's announcement by the Science and Engineering Research Council that it is now embarked on finding fields of research or important

research institutions, from which it can withdraw support. The plan is to find things to drop by early next year, and no later than April. Meanwhile researchers who may with gratitude have been allowed to retire from academic committees brooding on the future of their universities will now find themselves sucked up into another round of endless meetings, this time about research support. Before the dust has settled, there will be a great deal to be said on the important and daunting questions underlying the new round of enquiries.

In the past few years, the much-vaunted British system for supporting academic institutions, the dual-support system, has come to resemble a pair of millstones between which the research enterprise is being ground. Theoretically of course, there should now be no immediate problem. The contraction of the universities has come to an end, the British Government has been hinting that henceforth "level funding" will apply, while the government has in strict monetary terms kept its prime minister's promise (in 1980) that the civil research budget would be "protected". The snag is that constant budgets for the universities in present circumstances imply a steady drain of 0.5 per cent a year in real resources, while the constant research budget (spent through the research councils) has now to cover a greater range of activities, including payment of pensions to people whose services are no longer required. No wonder that the University Grants Committee is threatening (but some will say promising) greater selectivity in support of particular universities, and that the research councils are having to draw in their horns.

With this in prospect, any sensible British university should take a few simple protective steps. First, it should set up a research committee to attempt to decide on its priorities. The inescapable reason for doing this is simply that the universities were told to do just this two years ago, in the report of an investigation in whose existence the grants committee had a hand. But there is also a chance that such an arrangement would give the universities a capacity which they at present lack for knowing and then for understanding the range of research that their academics at present conduct. (In passing, it might be acknowledged that part of the trouble underlying British academic research in recent decades has been the indifference of universities to their own achievements.) And there is no doubt that such an understanding will be essential eighteen months from now, when the men from the University Grants Committee set out with their scalpels.

To be safe, however, universities should be careful to distinguish between academic research and the now fast-growing volume of industrial contract research carried out within universities (the once watchful eyes of the revenue authorities temporarily blinkered by the climate of the times). To say this is not to echo old-fashioned notions about the relative merits, intellectual and otherwise, of the two kinds of activities but is simply to say that managing academic and industrial research are different activities, often calling for different people. And while there is every sign that the British Government (and thus, to some extent, the grants committee) will applaud those institutions that earn a large proportion of their income from contracts, there is as yet no sign that the grants committee will relax its traditional standards of scholarship when choosing between the sheep and the goats. Moreover, that process cannot now be long delayed.

For the institutions responsible for managing the impending change, the grants committee and the research councils, the benefits of acting quickly are now overwhelming. The grants committee wants to concentrate research at a smaller number of universities within a system accommodating as many students as at present, while the research councils must somehow shed a large proportion of their fixed overhead cost, that represented by their commitment to research institutes and the people there employed. In an ideal world, they would put their heads together and recognize that their problems would more easily be solved in concert than separately. The only evil consequence would be that the goats (or is it the sheep?) among the universities would more quickly know where they stand — and that a greater proportion of them would be wrongly classified. Such a recognition of common sense may be the next threat to the universities.

Animal crackers

Using the cloak of animal rightists' groups to persecute people is wrong.

IN the pathology of personal vindictiveness, the way in which an unidentified resident of Pennsylvania has apparently set out to damage Dr Robert Weinberg, a professor at the Whitehead Institute at Massachusetts Institute of Technology, breaks alarming new ground. Last month, researchers in Britain as well as the United States were circulated with an anonymous notice, purportedly from an organization called the Animal Liberation Front, which uttered unspecified threats at those in cancer research who use animals in their experiments, but which singled out Dr Weinberg for having presented at *Nature's* September conference in Boston a paper supposedly encouraging painful animal experiments. The title of the paper was given as "Neoplasms as uncontrolled regenerative hyperplasia", which will have seemed a little odd to the many who know Dr Weinberg's work. To suggest that his activities entail more than the most marginal dependence on laboratory animals is, of course, laughable, which is the spirit in which the incident was reported a few weeks ago (*Nature* 4 October, p.401).

Since then, the plot has thickened. Whoever circulated the anonymous notice seems also to have been circulating scientific papers supposedly written by Weinberg. One curious feature of these documents is that they appear not to involve the kinds of experiments with animals that would send the animal welfare people into a frenzy. Weinberg has discovered that the fake paper he was said to have read at the *Nature* conference (at which, in reality, he gave a sober review of the recent molecular biology of oncogenes) had actually been mailed to him, under different authorship, several months earlier (when it had been filed together with other crank cancer theories). More mystifying is a letter, apparently widely circulated, that purports to be a manuscript submitted to the *New England Journal of Medicine* which puts forward a proposal for the use of steroid hormones in the treatment of breast cancer. An accompanying note says that the editors of the *Journal* would be grateful for comment. Again, there is no mention of the use of experimental animals.

There are several lessons to be learned from this curious chain of events, not the least of which is the discovery of how easily a researcher's attention may be distracted by malevolent and apparently pointless activity. It is, however, intolerable that productive people should be thus preoccupied. The best way to deal with nuisance mail of this kind is to tell the police, urge them to investigate and to forget about it all. That, unfortunately, is easier said than done, as will be verified by anyone who has personal experience of knowing there is somebody nursing some kind of hate. The calculation that the anonymous watcher is probably psychiatrically disabled is little comfort. Since Weinberg has not been the only target of the anonymous nuisance-monger's attention, it may be worthwhile that others affected should communicate with him or with *Nature*.

Second, while there is no serious danger that a well-established reputation could be shaken by the circulation of nonsensical papers, there is every chance that people's professional opinions could be altered in subtle ways by the circulation of, say, preprints masquerading as serious works. People should be on their guard against such dangers, unlikely though they may appear.

The use of the slogan "Animal Liberation Front" on some of the communications from Pennsylvania points to another hazard — the danger that ill-considered actions by people who should know better will provide a cloak beneath which unwelcome allies may have their fling. Of necessity, because it is a banner for often illegal activities, the Animal Liberation Front cannot be a formal organization, with a constitution and a membership list. The result is that anybody can join, gaining attention for private causes from the publicity that has been engendered by those whose interest is to break into laboratories and animal houses. The hazard is one of those attending the activities of all terrorist organizations, however well-intentioned.

US space research

Space station threat to science spending

AN enormous share of the US National Aeronautics and Space Administration (NASA)'s space science budget may wind up supporting the space station, according to some initial assessments by scientists advising the agency. Peter Banks of Stanford University, chairman of a panel looking into scientific uses of the station, said last week that \$400–600 million a year might be spent by "code E" — NASA speak for its science budget — on space station projects. The NASA science budget is now \$1,404 million, which goes to support both basic research, mainly at universities, and such major projects as the space telescope and the Venus Radar Mapper mission.

The space station programme, which received official presidential approval last January, will not be able to purchase scientific instruments or support research and analysis activities with the \$8,000 million tentatively approved for the project. All of these expenses with presumably be borne by code E. NASA officials say that \$8,000 million will buy a basic manned module, two accompanying unmanned platforms, and an orbiting manoeuvrable vehicle to transfer payloads from the station orbit to higher orbits.

Some scientists, particularly those interested in Earth observation experiments, say that a single polar platform cannot be large or flexible enough to meet their needs, which means that the potential cost savings from sharing power supplies and communications equipment on the space station platforms will not be realized by the science budget. NASA appears to be resisting suggestions that a flotilla of small platforms be built in place of the large platform.

NASA has just sent out its request for

proposals for initial design studies of the station, and the specifications include the two large platforms. Contractors for the initial design work will be chosen on 1 April next year. (John Hodge of the space station programme told Banks's committee this summer that the large platforms provided a more "unified" approach to the programme and would be well suited for commercial projects, such as manufacturing in space.)

Despite such concern, space scientists are reluctant to criticize NASA's space station plans. The frequently heard argument is that, because the space station is going to be built in any event, the best thing scientists can now do is to offer their full support. In that way, they can hope to influence some design details to ensure that some good science can be carried out.

Burton Edelson, director of the Office of Space Science and Applications, told the Space and Earth Sciences Advisory Committee last week that, because his office had been "supportive" of the space station over the years, it had already been able to make the project "more suitable" for science. He said there would not have been unmanned platforms at all if it were not for his office's advocacy.

At the same time, some say that there are great political pressures on the NASA science programmes to make use of the space station, which is seen by many as long on engineering and short on purpose. The same reservations were expressed, to a lesser degree, about the role of science in the shuttle programme. The Office of Space Science and Applications is spending 13 per cent of its current budget on shuttle projects.

The Banks committee, significantly, was not asked to decide whether scientific

activities could better be carried on the station, but only what activities could be carried out on it. It seems agreed that the space station opens up some new possibilities but most of the experiments listed by Banks's committee, and by the Space Science Board in a separate study, could clearly be done as well if not better without the station.

Some relief for the science budget may come from abroad. NASA administrator James Beggs has been pushing hard for Japanese and European contributions to the cost, to the tune of \$1,000 million and \$2,000 million respectively. There is some talk of the foreign contributions going for a second polar platform.

Stephen Budiansky

ESR to Grenoble in Franco-German deal

Paris

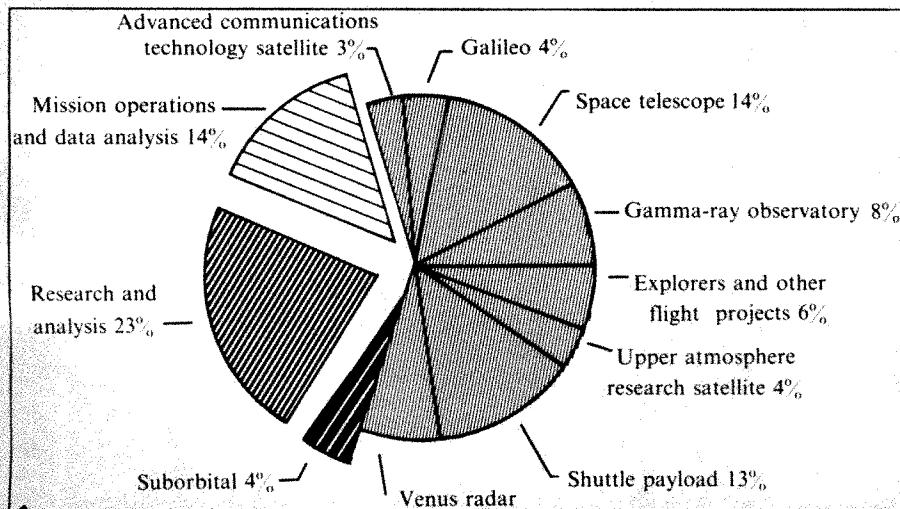
OFFICIALS representing Hubert Curien and Heinz Riesenhuber, the French and German science ministers, last week had to answer for the Franco-German decision to site the European Synchrotron Radiation Source (ESRS) at Grenoble.

On Friday in Brussels, they met their opposite numbers from the smaller European states under the auspices of the European Science Foundation (ESF) "progress committee" which — according at least to Denmark and Italy — had been gathering and evaluating site proposals. Instead, the committee was faced with a decision by France and Germany that ESRS should be built in Grenoble.

In fact, the first notice other governments had of the "decision" — except for press reports — came in a joint letter from Curien and Riesenhuber only on Tuesday last week, too late for official reactions by Friday. What may now emerge is an agreement between France, Germany, Britain and Italy to which the smaller states, notably those of Scandinavia, might adhere in a separate consortium. But Denmark, clearly the most hurt is unwilling to participate. The Netherlands is also playing cool.

Meanwhile, Britain, which France and Germany dearly want as a partner, while strongly backing the Grenoble site, has no money to offer, and may enter only with observer status "or something between that and full participation". The French certainly are not keen to see ESRS start up as a purely Franco-German institute, as the Institut Laue-Langevin was before 1967.

And why did France and Germany (and in effect the United Kingdom) ignore the ESF progress committee? Because it had too low a political level, say French sources, and would have been unable to trade one facility for another. This, finally, is what France and Germany did with ESRS and the trans-sonic wind tunnel, now to be built at Cologne. Robert Walgate



Europe in space

Italy treads Columbus path

US pleas for European collaboration in the planned space station have won hearts and minds in Italy. The Italian minister of research, Mr Luigi Granelli, was in London last Friday to persuade the faint-hearts of the British Government that they should contribute some £40–50 million a year from 1986. For Granelli, Europe's only future lies in space. "We've fallen behind in electronics and in biotechnology; we just can't afford to fall behind in space as well." His own government appears to agree. But Britain is still counting its pennies.

Mr Granelli's enthusiasm may yet work in sceptical London the magic it has worked in Rome. Earlier this year, seeking an increase in the budget of the "national space plan", Granelli told his cabinet colleagues that they should either cut the annual 50,000 million lira (£20 million) plan to nothing, or increase it four-fold. "It would have been a waste to continue spending just the 50,000 million lira" he said last week. The Italian cabinet seems to have agreed, to have pulled down their visors and taken a walk in space with Mr Granelli. From this summer, Italian space spending has risen to 150,000 million lira, and will reach 200,000 million lira next year (see box). Italy's contribution to the European Space Agency (ESA), at present 120,000 million lira, comes on top.

Safety in numbers

THE increased Italian space budget is mostly to be spent on technology, but X-ray astronomers may just benefit. The package includes approval for a "phase-B" detailed design of an X-ray satellite (SAX), Italy's first, due for launch at the end of the decade. Italian astronomers have been seeking outside collaborators on instruments for SAX which already, according to some observers, has too many small instruments incorporated to satisfy this or that hungry group of Italian astronomers.

International collaboration could stretch SAX further. Discussions are now at a critical stage with Dutch high-energy astronomers, but Britain and West Germany have been occupied with their collaboration in the German-designed ROSAT. Now, however, Italy may face a problem with the Dutch Government, which has set its face against further satellites after last year's infrared satellite IRAS.

Apart from SAX, other items on the Italian agenda include ITALSAT (a telecommunications satellite) IRIS (a Space Shuttle upper-stage that would put SAX in orbit), TSS (a tethered "satellite" to hang in the atmosphere 100 km beneath the Shuttle) and LAGEOS, a geodesy satellite (also launched by an IRIS-Shuttle combination).

Robert Walgate

Granelli urged the same argument last week on British industry and information technology minister Geoffrey Pattie. Granelli is convening the first ministerial meeting of ESA since 1977 in Rome next January to plan Europe's response to the US invitation. He hopes that British ministers will not be dragging their feet.

Europe needs "coherence" and "a vision" in the face of the National Aeronautics and Space Administration (NASA)'s plan to begin detailed design of the space station (phase B) on 1 April, 1985, and to start construction in January 1986. A series of bilateral arrangements with NASA would be dangerous, he believes, so he seeks a 70 per cent increase in the ESA budget in the period 1986–91.

The Italian vision, shared by West Germany (see *Nature* 25 October, p.696) is that of Columbus, and adjunct to the space station consisting of a manned laboratory (developed from Spacelab), an experiment platform and a control module. But Granelli, statesmanlike, says he is more interested in principles than details. There are as many technical proposals for collaboration as there are space technicians in Europe, he admits. Whether or not Europe builds Columbus matters less than that the old continent should safeguard "our standing and negotiating power with the United States".

In Britain, the issue appears to be whether Europe should plan a truly independent manned capability in space (as seems to be the view in Italy, West Germany and France), almost without counting the cost, or whether the approach should be more gentle. Nevertheless, it is stressed in London that "Britain wants to do something European". There are fears that mainland Europe is beginning to write off Britain as a potential partner. Collaboration in the space station may be a test.

The effect of all this on the space science programme is cloudy. Granelli said last week that he did not know what would happen to the recommendation that ESA's mandatory space science programme should increase by 40 per cent by 1990, mostly for astronomy.

In Britain, cabinet sources suggest that cash for any space station collaboration should come out of "a political pocket", leaving existing space sciences unaffected, but Granelli is not so sure. "When you talk of science, you must think beyond the existing community of space users." Granelli was thinking of "science in zero-gravity" — materials processing and so on. But he added that Earth scientists and astronomers now using space through ESA should not "in principle" suffer, although "someone has to make a choice in the end." So something may be sacrificed on the altar of the space station.

Anglo-French cooperation

New guise plea

FRANCE and Britain should set up a joint foundation with the aim of linking universities and industry, the French President, François Mitterrand, told the British Parliament last week. A new idea? No. But each time it pops up it takes a slightly different shape, and each time Britain knocks it down again.

The idea first surfaced in Edinburgh two years ago, during a meeting of the largely commercial Anglo-French Council, when the research director of the French oil and chemicals company Elf-Aquitaine proposed a joint research and development planning agency. This was to be heavily weighted towards industry, and was to devise more projects like the European Airbus.

That plan died until Robert Chabbal, a director at the ministry of research in Paris, transformed it into a proposal for a series of bilateral science and technology seminars. These have taken place — but between France and Sweden, and France and West Germany, rather than with Britain. The British view is that France has gained more than Sweden or West Germany from the meetings so far.

Meanwhile, the science counsellor at the French Embassy in London has been trying to convince his British colleagues that some kind of collaborative structure should be established between Britain and France, but without much success. The French view is that collaborations move best beneath a well-defined governmental "umbrella agreement". The anarchic British, however, say there are already plenty of routes for collaboration, and therefore no need for new structures.

President Mitterrand's intervention may have been stimulated by the suggestion by British science minister Peter Brooke, at the Council of Europe last month, that Europe should establish an international science academy. The French consider that this may be a sign of enlightenment in Britain, perhaps even a basis for their own idea of a science-industry forum.

The British are still reluctant, however, and suspicious that France merely wishes to tap British invention. They may be right. M. Guy Denielou, president of France's only technological university (Compiègne) said last week that he fully approved of an institution such as Mitterrand had proposed.

"Of course, I would hope to be a customer" he said. "British research is too big for British industry... All your ideas get taken up in America or Japan." It would be a good thing, said Denielou, if British science could feed some ideas into French industry as well. The same thing could also happen in reverse, he said. But the suspicious feeling in Britain, rightly or wrongly, is that the traffic would work out

Chinese nuclear power

Japan sets sights on business

Tokyo

THE chief obstacle to an atomic energy cooperation pact between China and Japan, the unwillingness of China to permit verification of non-military uses, seems likely to be removed in the coming weeks. China is now apparently prepared to allow some sort of inspection of nuclear plants supplied by Japan to be carried out under the supervision of the International Atomic Energy Agency (IAEA).

The exact form that inspection will take is not, however, clear. On the Japanese side, Michiyuki Isurugi, minister of state for the Science and Technology Agency, was willing only to say that Japan had received a "new proposal" in response to its wish, repeated over four rounds of talks, that China accept supervision by IAEA or provide other concrete means of guaranteeing peaceful use of atomic energy facilities. Isurugi did, however, express confidence that he would be able to travel to Beijing in the next few weeks to sign a pact. The Chinese side seems to have been rather more forthcoming, according to an article in the *Asahi Shimbun* claiming that officials in Beijing had stated they were now willing to allow IAEA inspection.

Earlier this year, Japan had received promises that China would allow "goodwill" visits of Japanese experts to Japanese-built plants in China. This was enough for Mitsubishi to go ahead, with government encouragement, and win a ¥1,500 million (\$6.3 million) contract, against strong French and West German competition, for pressure vessels (using US Westinghouse technology) for one of China's first nuclear reactors. But lack of a full cooperation agreement was thought by the nuclear industry to hinder its competition with the French, West German and British companies that have joined the rush to build nuclear power stations in China.

Much is at stake, for China is the last major nation without any nuclear power generating facilities and has committed itself to installing 10,000 megawatts of capacity — a minimum of ten reactors — by 2000. US companies, whose home market has dried up, should be the strongest in the field, but are for the time being sitting on the sidelines, waiting for the US-China nuclear cooperation agreement announced last April to be approved by (or at least submitted to) Congress (see *Nature* 309, 657; 1984). France, Italy and Brazil have already signed nuclear agreements with China — although none is so extensive as that signed by West Germany — and the Soviet Union has also indicated its willingness to sell reactors to China, apparently without assurances on strictly non-military use.

Meanwhile, construction has begun in China on two nuclear plants, one of 300 megawatts at Qinshan near Shanghai

which will use a Chinese-designed pressurized water reactor (PWR) with pressure vessels supplied by Mitsubishi, and the other with two 900 MW PWRs near Canton, which will supply part of its power to (and be partially financed by) Hong Kong. Final contracts for the second reactor have not yet been awarded but discussion has reached an advanced stage with Framatome of France for the reactors and the General Electric Company of the United Kingdom for the turbines.

Two more power stations are in the offing: one near Shanghai and a second in the northern Liaoning province. If US activity remains frozen, contracts for one of these could go to West Germany, to judge from Chancellor Kohl's optimism during his recent visit to Beijing.

When the US presidential election is over, though, the United States-China atomic energy accord is expected to be given high priority. If US companies get

back into the field, competition will be much more intense. But Japanese manufacturers still fancy their chances, because they have the best operating efficiency record in the world and believe China will go for reliability.

US commentators, on the other hand, have quoted Chinese officials as saying that they want to learn from the "teacher, not the student!" — virtually all of Japan's nuclear technology is derived from US designs. China is, however, almost certain to distribute its contracts among the advanced nations both to encourage cut-throat competition and to ensure that it can always ride out a sudden worsening of relations with one or more of its suppliers. Nor can the apparent willingness of China to allow inspection of Japanese plants be regarded as a signal that similar concessions will soon be made to the United States. Last April, when the possibility of "goodwill" inspection came up, the Chinese said they were taking into account Japan's unique position as the only country in the world to have been hit by nuclear weapons.

Alun Anderson

Atmospheric chemistry

Preparing for crises ahead

Washington

CITING "essential unanswered questions" about changes in the Earth's atmosphere, a US National Research Council panel has proposed a long-term international study of tropospheric chemistry, to be led by the United States. At a public meeting here, panel members agreed on the need to understand basic atmospheric processes in order to provide sound advice to policymakers and to avoid "crisis responses" to environmental problems. The panel's chairman, Robert Duce of the University of Rhode Island, said the proposed "Global Tropospheric Chemistry Program" could by the end of the century allow the impact of atmospheric changes to be anticipated.

Several panel members conveyed a sense of urgency about the unknown effect of documented atmospheric changes. Ralph Cicerone, the panel's vice chairman, said it was "shocking" how little is understood about cycling of atmospheric constituents. Dr Jerry Mahlman of the National Oceanic and Atmospheric Administration, an independent supporter of the programme, drew attention to increasing atmospheric levels of trace gases, especially methane, which could have an effect on temperature "equal to or greater than" carbon dioxide and which "can be expected to cause a 20°C cooling of the stratosphere within two or three human lifespans". Mahlman said nobody knows why methane levels are rising and that "we ought to find out".

The research council panel had been convened at the request of the National Science Foundation and the National Aeronautics and Space Administration.

The proposed study was generally welcomed, but there were some reservations about the apparent dominance of a supposedly international programme by the United States: the Global Tropospheric Chemistry Program is seen as a large component of the International Geosphere Biosphere Program recently approved in principle by the International Council of Scientific Unions (see *Nature* 4 October, p.402). Henning Rodhe, professor of chemical meteorology at the University of Stockholm, pleaded for a genuine international effort and reminded the largely US audience that "there is some atmospheric chemistry outside the United States".

The new programme would in its early stages concentrate on developing new instrumentation and classifying ecological habitats by their effect on the troposphere. Later research would focus on biological sources of tropospheric constituents, global distribution of trace gases, studies of photochemistry and investigations of removal processes. New global models would incorporate scavenging and chemical reactions in cloud droplets.

There has so far been no serious attempt at costing, but, according to one "very crude" estimate, the \$10-20 million spent annually on tropospheric chemistry in the United States would need to be doubled to get the programme started. Detailed proposals are to be drawn up at a series of workshops organized by Dr Cicerone. But the panel acknowledged the difficulty of persuading Congress to support a programme unlikely to produce useful results within 10 years.

Tim Beardsley

Cancer mortality

US half-rate goal in dispute

Washington

THE National Cancer Institute (NCI) continues to defend its "achievable" goal of halving US age-adjusted cancer mortality by the year 2000, first announced (to great dramatic effect) before the US Senate appropriations committee in April this year. But NCI has yet to make public any detailed justification for this claim, and its feasibility has been questioned. NCI plans to publish a full description of its year 2000 programme in 2 or 3 months, after review by the National Cancer Advisory Board.

Since 30 per cent of the 440,000 deaths from cancer each year in the United States are attributable to cigarette smoking, all agree that curtailing smoking is crucial. NCI's provisional estimate is that it should be able to halve lung cancer mortality by 2000 by preventive measures alone. But there are doubts whether NCI can achieve the 50 per cent reduction in smoking over the ten years to 1990 needed for this target.

As a government agency, NCI is prevented from lobbying for what is known to be one effective means of reducing smoking: higher taxes on cigarettes. NCI's weakness on this score was underlined in the summer, when Congress voted to lower cigarette taxes as part of a compromise that included strengthening the health warning on cigarette packets. For the same reason, NCI cannot take a position on, for example, banning cigarette advertising or restricting smoking in public places. And NCI's own advertising efforts are limited to free "public service" air time donated by radio and television stations and pamphlets distributed in physicians' waiting rooms. The emphasis is on helping only those who want to stop smoking, rather than encouraging confirmed smokers to reform.

Even if smoking could be halved by 1990, success in halving lung cancer by the end of the century would be far from assured. While smoking appears still to be decreasing among males, there are signs that it is increasing among females, and such reductions in smoking as there have been in recent years have been restricted to the higher socioeconomic classes. In order to reach the target, the NCI programme would have to be effective among the older/heavier smokers who are most at risk but the hardest to convert, according to Dr John Higginson of Universities Associated for Research and Education in Pathology. Dr Lewis Kuller, director of the department of epidemiology at the University of Pittsburgh, goes further, saying that the year 2000 goal on lung cancer will be achievable only if a chemopreventative agent is discovered for treatment of those who have stopped smoking.

The target is defended by Dr Joseph Cullen of NCI. The institute is about to embark on large-scale intervention trials

that will include 10 million subjects over 5 years, designed the better to define how to persuade people to stop smoking. There will be a special programme for high-risk groups, and physicians will be trained in anti-smoking counselling. Cullen predicts that NCI will extend its influence with policy-makers and the media, and that the continuing shift in public attitudes will be helped by, for example, boycotts against magazines that carry cigarette advertising. Cullen will not be drawn on how NCI will get around the statutory limits on its authority except to say that more federal and state funds will have to be used.

Smoking aside, NCI plans to achieve a further 15 per cent reduction in cancer mortality by the year 2000 by ensuring full public access to state-of-the-art treatment. Dr Peter Greenwald, director of NCI's division of cancer prevention and control, says this target makes no allowance for advances in treatment yet to be discovered or for earlier detection, but that it is based on treatments now at the stage of clinical trials. On top of the reduction due to improved access, Greenwald anticipates another 15 per cent reduction due to improved treatment of micrometastases; he points to experimental treatments using monoclonal antibodies targeted at specific oncogene products. While acknowledging that the figures might be disputed by a conservative statistician, Greenwald stresses the importance of having goals.

Others doubt whether these dramatic predictions can be defended. While more widespread screening programmes could markedly reduce breast cancer mortality (possibly by up to 30 per cent), and perhaps cervical cancer also, the other major killers seem stubbornly resistant to improved treatment. Kuller says he "would have difficulty being convinced" that these improvements are in prospect without further major advances in early detection. Tests for cancers of the gastrointestinal tract, for example, are still much needed.

Perhaps the most controversial element of NCI's year 2000 plan is the goal of reducing mortality by 5 per cent by changing eating habits. While inter-population comparisons indicate that up to 35 per cent of cancers may be caused by dietary factors, the precise components involved are not known. Despite the small number of studies of fibre intake and cancer, NCI's director, Dr Vincent DeVita, told the Senate Appropriations Committee in April that a doubling of fibre intake could reduce colon cancer by 30 per cent by the year 2000. NCI also now believes that a reducing fat consumption will help.

Others are not so sure: Kuller says the 5 per cent target "could be wishful thinking". In 1982, the National Academy of Sciences concluded that it was impossible to quantify the likely effect on

cancer incidence of dietary changes. Higginson believes the effect of diet on cancer incidence in adults has been exaggerated, and points out that the reductions in fat consumption seen in recent years, which have had a marked effect on heart disease, have so far failed to have the effect on cancer incidence that would be expected according to the simple dietary hypotheses, thus making the overall NCI target even less realistic.

Tim Beardsley

European environment

Hopes to reform farming

Brussels

THE European Community may be about to mount a vast new programme of research and development in agrochemicals. That, at least, is what will happen if the commissioner for environment and internal market affairs, Mr Karl-Heinz Narjes, gets his way. At a conference in Dublin two weeks ago, Mr Narjes argued that pesticides and fertilizers have had an undesirable effect on the environment, and that the time has now come to encourage technical innovation to yield more selective but less persistent pesticides.

The environment commissioner has been trying for some time to persuade his colleagues in the commission that environment policies should be integrated with others, and with agricultural policy in particular. Formally, this principle is written into the current action plan on the environment. Now there are signs that the member states are coming round to Mr Narjes's way of thinking.

Mr Narjes is not anti-farmer. Instead, his aim is that agriculture should continue to profit from the use of chemicals without putting an intolerable burden on the environment. In Dublin, he was pleading for more work on the toxicology of pesticides, allowance for regional and national differences of cultivation techniques and a training programme for farmers.

Within the Community, the Dutch Government has already advocated the use of European Community funds to protect the environment linked to farming in less-favoured areas. Meanwhile, the United Kingdom, backed by Ireland, has been urging that Community agricultural funds should be made available to farmers for "environmentally friendly actions".

The outlook for Community regulation of agricultural pollution seems less bright, however. Member states have been unable to agree on a list of acceptable pesticides since 1976, while proposals for maximum permitted levels of pesticides in foodstuffs have been gathering dust since 1981. Mr Narjes seems bent on circumventing the regulatory log-jam by tackling the problem at source.

Anna Lubinska

Soviet meteorology

Bolts from out of the blue

Up to 25 per cent of recorded weather information fails to reach the Soviet Union's Hydrometeorological Scientific Research Centre, the new director, Aleksandr Vasil'ev, has told an *Izvestiya* correspondent. The interview is also the first indication that Vasil'ev had been promoted from his former post as deputy, thus generating speculation that his predecessor, Dr Mikhail Petrosyants, had been dismissed for failing to forecast the hurricane which caused widespread devastation in the Russian Federal Republic on 9 June.

The storm, apparently caused by a warm dry airstream from the eastern Mediterranean meeting a cold airstream from the Barents Sea, has cost the Soviet Union upwards of 120 million rubles (\$150 million) in restoration work, compensation and insurance. Devastation of houses and "production facilities" is reported from Ivanovo, Gor'kii, Kalinin, Kostroma and Yaroslavl as well as in the Chuvash republic, with an unspecified number of deaths. A special resolution of the party central committee and the Council of Ministers to make good the damage done by "a natural calamity" was passed in mid-July.

The Ivanovo hurricane was followed almost immediately by three further meteorological calamities — extensive flooding in the Baikal area in mid-June, a hurricane in the Volynia district in Western Ukraine in mid-July and flooding in the Amur basin "complicated by Typhoon Holly" in late August. But in these cases, although there was considerable damage to agriculture and production schedules, some warning seems to have been given. In the case of Ivanovo, however, there was apparently no warning.

Reliable weather forecasting is particularly important to the Soviet planned agriculture, where the logistics of sowing and harvesting are determined regionally. Moreover, Soviet meteorologists still have to live down the memory of last autumn, when the unexpected onset of severe weather left more than 40 ships of the Arctic mercantile fleet stuck in the ice.

In the event, the new icebreaker *Leonid Brezhnev* extricated the vessels. The Soviet press at the time stressed the valuable lessons learned for future Arctic navigation, which may be why the northern navigation season has been ended several weeks earlier this year. But *Pravda* also noted a year ago that Arctic weather was not by itself "guilty" of causing the emergency, and that all those responsible for the ships' presence in the ice so late in the season should share the blame. Coming only a few months later, the Ivanovo hurricane may well have made it necessary for the meteorological service to offer a scapegoat. And, at 65, Petrosyants is

But is Vasil'ev justified in blaming the problem on poor information flow? The question is pressing because of the new drive to extend the use of "improved" land, which has to be made fit for agriculture by either irrigation or drainage. Last week's central committee plenum admitted that all is not well with the much publicized food programme, launched by Leonid Brezhnev only in 1982, and "further measures" were urged by Mr Chernenko including a major extension of irrigation and the bringing in of virgin land, in Soviet Central Asia. River diversion projects, allowed to lapse during the Andropov era, seem on the way back. Yet even the most optimistic planners admit that such schemes can only yield the projected harvests if every drop of irrigation water is carefully husbanded. Failures in weather forecasting could have serious results.

The Soviet meteorological service

appears to have the technical means. It maintains a union-wide network of weather-watch stations, including "out-posts" such as that on Franz Josef Land, and a fleet of weather ships, which not only participate in forecasting programmes but also attempt to correlate Soviet weather conditions with such factors as the change of sea temperature in the northern Pacific. As early as 1970, experiments were under way with networks of automatic recording stations which would feed data automatically into a computer.

Reliable weather forecasting is also one of the main justifications for the Soviet space programme, while the export of meteorological equipment and the training of operators form an important part of Soviet aid programmes to developing countries. Vasil'ev's suggestion, that it is not the information gathering but the information transmission system which is at fault, is one to which the State Committee for Hydrometeorology and Environmental Control will no doubt pay attention.

Vera Rich

Corporate research

Monsanto goes for independence

St Louis, Missouri

THE Monsanto Chemical Company's new \$150 million Life Sciences Center in Chesterfield, St Louis, opened this week and will employ 1,200 scientists, engaged mainly in basic biological research, by 1987. About one third of the scientific budget of the company is earmarked for biotechnology research, and Richard Mahoney, Monsanto's chairman, is prepared to invest \$25–\$75 million before seeing a return in the marketplace, at least ten years from now.

The first commercially-produced biotechnological product will be a bovine growth hormone (methionyl bovine somatotropin, MBS) which will increase milk yields by 15–20 per cent, thus allowing farmers to reduce the sizes of their herds. But the blue-suited Monsanto managers are most proud of their company's commitment to basic research, in which they claim now to be self-sufficient. The basic biology of the MBS development had to be contracted out to Genentech.

Monsanto will, however, continue supporting university research programmes, including the 5-year \$23 million collaboration with Philip Needleman's group at Washington University (St Louis). Here, the discovery in the mammalian heart of atrial peptides affecting sodium balance and smooth muscle tone (*Science* 223, 67; 1984) has led to current investigations of the role of these peptides in human blood pressure control.

Roof-top greenhouses and 123 "growth chambers" at the new institute are the two most visible signs of the company's investment in plant genetics and physiology. In plants, expression of genes via the soil

bacterium *Agrobacterium tumefaciens* may eventually confer characteristics such as insect or disease resistance and increased seed production; now Monsanto is extending its earlier work on pea plants (*Science* 223, 838; 1984) to petunia and tomato. The commercially attractive goal of expressing genes in monocotyledonous plants is, however, well advanced in European laboratories; altered *A. tumefaciens* plasmids can be expressed in asparagus (see *Nature* 311, 594; 1984) and plants of the Liliaceae and Amaryllidaceae families (*Nature* 311, 763; 1984).

At last week's opening ceremony, Frank Press, president of the National Academy of Sciences, endorsed Monsanto's research investment, but warned of the dangers of an imbalance between federal and industrial research spending. (Industry now spends more on basic research.)

Press attacked the government practice of supporting work by a single investigator in a single department, and stressed the need for a different basis for the US grant system. Monsanto is ideally placed to make the massive financial investment needed for a multidisciplinary attack on scientific problems and says it intends to continue publishing its research promptly in the scientific literature.

It claims to have learned from past mistakes in the arena of public opinion, being eager to comply with the National Institutes of Health guidelines for recombinant DNA research and preaching the benefits of biotechnology research to the public. So long as it remains open about its work in this highly competitive area, this huge boost to basic biological research will

Spanish universities

Reforms beginning to work

Barcelona

SPANISH universities are in a state of upheaval. The effects of last year's Law for University Reform are making necessary important changes in the structure of universities, affecting in particular the teaching staff, the number of departments, the statutes of the universities and their control.

Just before the end of the academic year, steps were taken to reduce the number of professors under contract and to give some of them tenure (see *Nature* 308, 395; 1984). Commissions were appointed for each discipline, which then decided who qualified for permanent positions. The results now published show that around 6,000 out of 8,000 candidates qualified, a proportion that the Ministry of Education believes accurately reflects the situation.

Mount Wilson Inc.

Washington

A CALIFORNIA industrialist has formed a corporation to take over the Mount Wilson observatory. The present owner of Mount Wilson, the Carnegie Institution of Washington, which also operates the Las Campanas observatory in Chile, announced this summer (see *Nature* 15 August, p.88) that it can no longer afford to keep both sites and will shut down Mount Wilson on 1 July 1985 unless a new owner can be found.

The new corporation, called the Mount Wilson Research Corporation, was founded by George Roberts, who made his fortune designing and manufacturing aircraft lavatories. His company, INCA Corporation, also made the zero-gravity lavatory for the space shuttle.

The corporation has no funds as yet, but hopes to raise an endowment of several million dollars, earnings on which will provide the basic annual operating costs of the observatory. Foundations and individuals are being approached for contributions.

According to Dr Arthur Vaughan, one of several research astronomers associated with Mount Wilson who are involved in Roberts's effort, the corporation is talking to universities and organizations such as the Smithsonian Institution and the Jet Propulsion Laboratory about entering into a consortium.

Vaughan said that the aim is both to maintain an in-house research staff at Mount Wilson and to serve the needs of outside researchers. To that end, all principal investigators would be involved in managing the use of the facility. The board of directors is to be made up of non-astronomers who will be concerned largely with the financial health of the corporation.

Stephen Budiansky

The results varied considerably between the different commissions, and although there was no obvious difference in the quality of the candidates, acceptance rates varied between around 50 per cent and more than 80 per cent. Those not selected are preparing to do political and legal battle. There will be more positions next year, and the situation of university professors will have changed profoundly in a period of only two years. Some people fear the long-term effects, particularly because the number of students is beginning to decrease and there is a danger of saturation of university positions soon.

The statutes of universities will have to be adapted to the new regulations. All universities have been asked to make proposals, and these will have to be approved by the government. The body in charge of this task is the General Assembly (Claustro) which includes professors, students and administrative staff. In some universities, conflicts have arisen between diverging interests.

An important change in the structure of universities, which will be reflected in the new statutes, is the definition of university departments. There is widespread agreement that the present departments are too small and too numerous, having mostly been built around a single professor. It is hoped that the new regulation will stimulate the concentration of people, administration and equipment in order to build up more efficient structures.

From now on, departments should have a minimum number of professors (twelve in the latest version of the law), and a full-time professor as the head of department. Normally, there should be only one department in any subject in a single university. The names of the departments should in principle correspond to areas of knowledge defined by the ministry. In some cases, these regulations may cause problems, for instance in medicine and engineering faculties where many departments have mostly part-time professors or in departments where the members are physically separated or working in very different research subjects. In any case, the power of full professors ("Catedráticos") will probably be diluted in these bigger departments.

The most important change is that the national government is transferring control of the universities to the autonomous governments of the different regions of Spain. Agreement to hand over the universities to the Government of Catalonia has already been reached. But strong differences still exist about the amount of money to be transferred and, as a consequence, the process is at a standstill. The government hopes that this period of upheaval will result in more dynamic universities.

Pedro Puigdomènech

Star wars killings?

Washington

ALTHOUGH almost all of the \$1,300 million budgeted this year for the Strategic Defense Initiative (star wars) is committed to research projects that have been under way for some time, defence contractors are already sensing a kill. "The Department of Defense has budgeted \$24 billion over the next five years for the Strategic Defense Initiative", reads an announcement from the American Society of Mechanical Engineers (ASME). "Will you find a place in this state-of-the-art cornucopia — expected to be a greater undertaking than the Apollo program?" To help them out, ASME is sponsoring a day-long seminar in Washington in mid-November, featuring briefings from key defence officials to show "how to get in on the ground floor". The admission fee is \$245 a head.

The only new funds to come out of the star wars programme so far are \$6 million to be awarded for an initial "creative effort to develop methodologies for defining alternate architectures". Proposals are due by 1 November; it is expected that more than one contractor will be chosen. The winners will be asked to "develop the strategic defense mission", considering both "treaty constrained and unconstrained threats", and to study various "candidate" anti-ballistic missile technologies.

Stephen Budiansky

Balzan prizes



PROFESSOR Jan Hendrik Oort of Leiden University in The Netherlands (above) has won this year's Italian Balzan Prize for astrophysics. The genetics prize has been awarded to Sewall Wright (95), professor emeritus in the Universities of Chicago and Wisconsin. The winner of the prize for history and criticism of the literatures is Professor Jean Starobinski of the University of Geneva.

Each of the winners will receive 250,000 Swiss francs. In 1985, the three prizes will be awarded for mathematics, palaeontology and history of occidental art.

North Sea pollution

West Germany calls for action

Brussels

WEST Germany faces other North Sea coastal states in Bre en this week with demands to make headway in the next two years on marine pollution and improved monitoring of the ecosystem of the valuable fishing grounds of the North Sea.

Participating in the International Conference on the Protection of the North Sea are ministers from the coastal states (Belgium, Denmark, France, the Netherlands, Norway, Sweden and the United Kingdom), from whom West Germany hopes to extract a political commitment to ratify and strengthen existing international conventions. The European Economic Community is also invited.

The North Sea is at present covered not by one but by several international conventions governing the protection of the marine environment from pollution from land-based sources (Oslo, Paris), from pollution by oil (Bonn) and from dumping (London). Not all have been ratified.

The West Germans stress the danger of latent chronic pollution (accumulation of chemicals) as well as more directly visible pollution such as oil. They are particularly concerned about the ecological sensitivity of coastal waters, river estuaries and mudflats, important feeding grounds for fish and birds.

The discharge of dangerous substances through inland and coastal waters is controlled by the 1974 Paris Convention. In 1976, the European Community also adopted a framework directive on emission standards for industrial waste discharges, but so far only limits on mercury, cadmium, polychlorinated biphenyl wastes, waste oils and lindane (last June) have been adopted, out of a list of 129 dangerous substances.

According to Greenpeace, the environmental pressure group, 1,120 tonnes of cadmium and 1,000 tonnes of mercury continue to flow annually into the North Sea. Twenty-three million tonnes of toxic sludge end up in Rotterdam harbour.

Some 11 million tonnes of largely untreated sewage sludge also end up in the North Sea, with the result that algal growth in the North Sea has increased considerably over the past 5-10 years, according to the West Germans.

The West German proposals also include a system for licensing wastes, discharges, and international information exchange on the quantities discharged and requirements that the pollution load should be reduced. At the beginning of this year, West Germany introduced a domestic system of fines for pollution of the North Sea.

According to the West Germans, there is a definite need for further action, including better enforcement and an extension of the Marpol conventions. They also propose that the North Sea be designated as a

special area, that sea dumping should be reduced and eventually dropped. The European Community is preparing a directive banning the dumping of black-listed substances and limiting others.

To combat oil pollution, West Germany proposes a region-wide system of airborne surveillance, improved cooperation in port state control, more use of deep-sea pilots' services and greater compliance by drilling rigs with rules the oil content of effluent.

West Germany also wants to extend research and development programmes to include more biological monitoring and the sampling of more substances. The Paris Commission (the secretariat of the Paris Convention) already monitors mercury, cadmium, polychlorinated biphenyls, oil

from refineries and reception facilities and drilling platforms as well as radioactive effluent from processing plants. The Community intends to start monitoring titanium dioxide wastes next year. West Germany suggests that all this information should be collected in an international database.

Community support for the West German proposals is important, if only because Community directives are binding on member states, whereas conventions enter into force only when ratified. It is therefore unfortunate that, in the case of the draft directive on accidental marine spills of oil and other dangerous substances, now under discussion, West Germany, together with Belgium, Denmark, the United Kingdom and France, feels that a Community directive is neither "necessary nor desirable".

Anna Lubinska

No canker cure from China

Washington

US DEPARTMENT of Agriculture (USDA) scientists working to combat the citrus canker now rampant in Florida (see *Nature* 27 September, p.293) are frustrated by a ban on new agricultural science exchanges with China. The US scientists were hoping to use a symposium planned for this month in China as an opportunity to collect new citrus germ plasm, possibly including canker-resistant strains. But the symposium has now been called off, and researchers are wondering whether the ban will be lifted in time to plan a visit for 1985.

The ban was ordered a year ago by the under secretary of agriculture for interna-

tional affairs, Daniel Amstutz, in retaliation for a Chinese decision to cut imports of US grain, breaking a contract. At the time it seemed likely that China would suffer more from a ban than the United States. The Chinese action was in turn a retaliation for US limits on textile imports.

Southwest China is believed to be the region where both citrus plants and citrus canker originated, according to Dr Jack Hearn of USDA's horticultural research laboratory at Orlando, Florida. American and Chinese scientists last year exchanged plant materials of interest, but citrus canker had not then appeared in the United States.

Tim Beardsley

Whaling

Japan pleads to carry on

Tokyo

THE Japanese Government is to try again to persuade the United States not to retaliate if Japan breaks the whaling quotas set by the International Whaling Commission (IWC) earlier this year (see *Nature* 18 October, p.595). Talks will be held in Washington on 1 and 2 November between the director-general of Japan's Fisheries Agency, Hiroya Sano, and John Byrne, director of the US Department of Commerce's National Oceanic and Atmospheric Administration. Earlier attempts to reach agreement ended in failure on 16 October.

As a result, the Japanese sperm whale coastal fishing fleet (owned by the Nihon Hoge and Nitto Hoge companies) put to sea on Friday 19 October. By the following Wednesday, however, before they had had a chance to catch a whale, the four boats were ordered to return home to give new talks a chance of success.

IWC set this year's sperm whale catch quota at zero, in the absence of agreement among the member countries. Japan,

which had been expecting a minimum quota of 400, vital to keeping its coastal industry going, thus considers it has been unfairly victimized by pressure from conservationists elsewhere.

The Antarctic Ocean minke whale quota was also cut, this time on a scientific recommendation, to a level that would give Japan a share amounting to 1,941 whales — some 600 fewer than the number at which Nippon Kyodo Hoge, Japan's only far-seas whaling company, says it can sustain commercial operations. The company is not yet abandoning whaling, however, and its mother ship, the *Dai San Nisshin Maru*, set sail from Yokosuka for the Antarctic on Saturday 27 October. It hopes that an agreement can be worked out with the United States before it reaches its catch quota around next January.

If the United States stands firm, then the Packwood-Magnuson amendment will be invoked if Japanese boats break the IWC rulings. Japan's fishing quota in the United States 200-mile zone could then be cut to zero.

Alun Anderson

Seascale hazard

SIR — The letter from Pomiankowski (*Nature* 13 September, p.100) raises two points which he considers introduce doubts about the conclusions of the report of the Black Advisory Group into the possible increased incidence of cancer in West Cumbria.

The first concerns the appropriate index on which to rank the occurrence of lymphoid malignancy in the 675 electoral wards of the Northern Children's Cancer Registry region. The suggestion is that the report was incorrect to include a table showing the wards with the highest incidence rates per 1,000 children, and that using instead the associated Poisson probabilities would have been more appropriate. It is important to recognize that these two sets of figures are not simple alternatives, since they measure different aspects of the disease frequency. The rate per 1,000 is a direct measure of the proportion of children under 15 years of age registered with lymphoid malignancy over the period 1968–82, which gives 9.73 per 1,000 in Seascale — calculated on the basis of a total of four cases during the 15 years and the 1981 census population. The cumulative Poisson probability, on the other hand, is a measure of the chance of finding the observed rate (or an even greater one) if the true (rather than the estimated) local ward rate were the same as the overall regional rate of 0.61 per 1,000.

The first index is an assessment of the average risk of a child in the area developing lymphoid malignancy before 15 years of age, whereas the latter is an indication of how unlikely it is that the raised rate in Seascale could have occurred by chance under a particular hypothesis. Each of these indices has its role and importance. In the case of a small-sized population and a small number of cases, the observed rate is subject to sampling fluctuation — and the associated Poisson probability helps to assess the size of this. Both figures are therefore informative and are given in the report. It is not true, however, to refer to the latter as representing the "incidence" of lymphoid malignancy — as was done by Mr Pomiankowski.

The second point concerns two childhood cases of lymphoma in Seascale registered since 1982. It is said that the exclusion of these from the calculations of rates and Poisson probabilities is "disturbing". The figures given in the report were provided by Dr Craft and his colleagues from the Northern Children's Cancer Registry for the years 1968–82. At the time (late 1983/early 1984) when these figures were being prepared, it was too early to include comprehensive cancer registrations beyond 1982 over the whole Northern Region since there is inevitably some delay in the notification of cancer cases to the regional register and, thus, in the availability of complete annual records for inclusion in analyses. For this reason it

is premature to consider the effect of these two cases — known about because of the special attention to the village — on the ranking of Seascale: it remains to be seen whether or not further cases of lymphoid malignancy have moved other small communities up into higher rankings, either of rates or probabilities. No doubt Dr Craft and his colleagues will do this in due course, and make further assessment of the situation possible.

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Defining length

SIR — On 21 October 1983, the Comité International des Poids et Mesures (CIPM) promulgated a new definition of the metre as: the length of the path travelled by light in vacuum during a time interval of $1/299\,792\,458$ of a second¹. This new definition raises some fundamental and practical problems.

At the fundamental level, one must reformulate Einstein's second relativity postulate: "Light is always propagated in empty space with a definite velocity which is independent of the state of motion of the emitting body"². For Einstein, this was an empirical fact. Now, this has become a definition and the corresponding empirical fact is that the length of a solid body depends neither on its spatial orientation, nor on the inertial frame where that body is at rest. (For example, in Michelson's celebrated experiment, the length of the arms of the interferometer did not change as that instrument was rotated or as it was carried by the Earth in an orbit around the Sun.) It is easy to derive the Lorentz transformation law from the above postulate.

On the practical side, this new way of defining length implies that there must be two *synchronized* clocks at the extremities of the measured object, in order to determine the corresponding time interval. As synchronization is best performed by light signals², this effectively means that in order to measure the length of an object, one ought to observe the round trip time of a light signal and divide the result in two.

Moreover, the return trip of the light signal must follow precisely the path along which the signal was sent. This is because a closed path surrounding a nonvanishing area (with a suitable set of mirrors) yields a result depending on the direction of travel, if the body being measured rotates. The difference in travel time (clockwise versus counterclockwise) is³

$$t = 4\omega A/c^2,$$

where ω is the angular velocity and A is the surrounded area.

If this effect is ignored (the round trip rule is not even mentioned in the CIPM recommendations!) the circumference of the Earth at the Equator is 123.9m longer if

measured eastward than if measured westward.

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Red Sirius

SIR — The recent correspondence (*Nature* 6 September, p.8) concerning the colour of Sirius reminds me that in the ancient astronomy of Iceland, the star was called Loki's Brand, obviously because of its periodic disappearance into the "underworld", but also because of its flaming colour. This apparent colour-naming is therefore not just a Greek phenomenon, but something which doubtless has considerable significance.

NIGEL PENNICK

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Mount Wilson costs

SIR — Your report (*Nature* 15 August, p.88) on the Carnegie Institution's plans to sell the Mount Wilson Observatory understates the scientific importance of this facility and overstates its operating costs. The \$1.56 million budget for Mount Wilson listed in the Carnegie Financial Report — apparently your source for this figure — includes all astronomers' salaries, fellows' salaries and support operations both for Mount Wilson and for the Las Campanas Observatory that Carnegie operates in Chile. A California consortium hoping to operate Mount Wilson asked the director to estimate operating cost for Mount Wilson alone. A detailed estimate, made by our business manager, was produced: \$0.75 million per year. This includes the fraction of all engineering and support services spent on Mount Wilson, but of course not those devoted to Chile.

Many individual astronomers and a number of organizations within and outside the United States have inquired about obtaining all or part of Mount Wilson Observatory. The reason is that it is an excellent site for stellar and solar astronomy. Your implication that this work is less exciting than extragalactic astronomy simply reflects a bias, one that is certainly not shared by all astronomers.

At the recent (October 1984) European meeting on solar physics, the current Mount Wilson solar and stellar research was described as vital, and a resolution was adopted urging that all possibilities for its continuation be explored.

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Can Europe avoid the Space Station?

from Erhard Keppler

The European nations could easily be tempted into a very expensive alliance with the United States to produce a space station. But is it worth it?

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RECENTLY, there have been talks between the US space agency, NASA, and various European governments in which the American side has aimed to reach agreement with European countries to share in the proposed US Space Station project, in particular its costs.

The space station is supposed to be a large facility in a near-equatorial Earth orbit, about 300 km above the surface. It could be composed of many smaller units launched separately but docked together in space. Such units may, according to NASA's futuristic view, reach dimensions of hundreds of metres in length. One possible configuration of such a station is a long tube, bent into the circumference of a wheel with spokes towards a centre for stabilization and finally put into slow rotation to provide centrifugal forces in the tube to substitute for the gravitational force on the Earth's surface.

On this view, a space station would have a crew ranging from 10 persons at the beginning to perhaps 100 some years later. The first station would, according to NASA plans, become operational in about 1992, and could (again according to NASA statements) serve either as an operational base for launches of Shuttle-launched spacecraft into other orbits, as an Earth observation station or possibly as the first stage towards a production facility in space. These are the essentials of the arguments put forward in support of NASA's space station, the costs of which are established to be about \$11,000 million in the next eight years.

Because of the uncertainties attending such projects, these cost estimates may easily grow to remarkable numbers, as has been shown during the development of the Space Shuttle. NASA's offer to the Europeans is then to participate in the development and manufacture of a space station within a cost-envelope of several thousand million dollars.

The offer has put Europeans in a difficult position. First, Europeans, who had their first experience of manned spaceflight only recently in the first Spacelab flight, are unable as yet to make a sound judgement as to the benefits of this endeavour especially as the second opportunity for a Spacelab flight is not due until 1985. In any case, large numbers of flights are ruled out by the tremendous costs per flight, now more than DM100 million (£25 million). At the same time, it is to be assumed that the US side will not support an eventually suc-

science by providing them further support, frequent flight-opportunities, for example.

Second, Europeans are afraid that the United States may eventually achieve a major success by using their space station commercially (for satellite launches, production and so on) so giving US industry better conditions by, for example low charges, so that European companies might be put at a competitive disadvantage. Thus it may turn out that two-step launches (from ground to a space station and from a space station into geostationary orbit) are much cheaper than direct ascents. Then European space companies would have no chance to compete with US companies in the vital communications market. But such apprehensions have no real basis; they are games in sandboxes.

Third, the conglomerate Europe has so far not been able to find a common strategy in space research and space technology. European space efforts are rather the sum of some countries' space efforts or bilateral agreements.

The case against

What should Europeans do in this situation? What are they able to do? The following are some of the conditions that must be dealt with, and the consequences which must be considered.

- One remarkable argument for the US Space Station, analogous to European apprehensions, is based on the irrational fear that, because the Soviets may build a space-station and so gain advantages denied to the United States, the United States should also build one just in case.

- The Space Science Board of the US National Academy of Science has rejected NASA's plans for the space station, which military sources consider unduly vulnerable.

- Many European scientists reject the space station because it is scientifically uninteresting and because they are afraid that the high costs associated and with its development and operation will draw away resources which are urgently required for more reasonable research.

So what have we gained so far from manned spaceflight? What is the prospect of a firm and solid basis on which to move further in this particular direction? Interestingly enough, the most frequently heard word in this context is "probably", as in its frequent assertion that the

of a space station, as well as the biological/medical sciences and, last of all, also the Earth observing sciences.

Astrophysics is, however, readily excluded because the required accuracy of observations, demanding constancy of viewing direction, is unattainable from a space station, which will always suffer from vibration due to the motion of the astronauts, from impacts of dust and small particles of meteoritic origin and from thermal tensions and relief. Astrophysics as well as Earth-observation techniques can, however, live perfectly well with unmanned spacecraft, specially because such missions should preferably extend over many years and be carried out in eccentric or polar orbits that will always be forbidden to manned missions because of the radiation load that astronauts would suffer.

Materials science is said to be another candidate customer. Materials scientists have just started to investigate the effects of reduced gravity on various rather elementary processes, which are certainly of high scientific interest. But at this early stage, there is not even the faintest hint that it will be necessary to have production facilities in space. Semiconductors, for example, can be made sufficiently well on the ground; neither crystal imperfections due to growth under gravity nor contamination have so far prevented the industrial production of microprocessors or large memories. There are other limits, of course, but there are also other interesting technical possibilities which may become the starting point for new technological pathways that are perfectly well pursued on the ground. Studies under zero gravity may in any case be satisfactorily continued on rocket flights or from unmanned satellites. The recovery of probes from such spacecraft is a well-proven technique, widely used in military applications. There is no justification whatsoever to mount a costly new programme on such evidence.

Finally, the interest of biology in zero-gravity conditions has greatly lost its attraction in the past few years. Indeed, space medicine flourishes largely because there are astronauts.

Much of the enthusiasm for the manned space station as a zero-gravity laboratory seems to stem from ignorance of what has already been achieved. The new "space science discipline" of materials science is carried on by scientists who have never

do not know about the ingenious robots designed over the years by hundreds of scientists to investigate stars, planets, our Sun or the Earth's atmosphere. Automatic self-controlled sequences (such as opening an oven, removing a melted sample, replacing it by another, closing the oven and repeating the procedure) do not have to be invented afresh. Such simple tasks have been possible for many years.

A physical experiment provides answers to the question for which it was designed. If the answer is negative, another experiment will be set up in order further to attack the problem. The rule is that other questions have to be asked after certain answers have been acquired. It is a fiction to believe that such experiments require the presence of people. If the question asked by an experiment is clever enough, the answers will be "yes" or "no". To design such an experimental set-up so that it will not be disturbed by external measures is a challenge to the skill and imagination of the experimenter.

Passengers

The very presence of astronauts in a space station requires extraordinary efforts. More than half of the cost of the development of Spacelab could have been saved if the systems had been unmanned. But for manned systems, the effort will always be greater than is really necessary, for nobody will take responsibility for endangering people's lives. Efforts of this kind are concerned with the number of subsystems and their redundancy, with their reliability, with the selection of materials (beginning with their outgassing performance) and with endless functional tests, the complexity of which is a nonlinear function of their number. The very size of the system requires an enormous amount of testing; the Spacelab Manual, for example, is several thousands of pages thick.

The requirements of reliability, and the selection techniques and materials, result in extremely high costs for all on-board systems, including instrumentation. But parts ordered to the necessary high standards of reliability have prices higher by factors of 100 than those of commercially available parts. Many are only available in the United States, where they are produced mostly for military purposes. In contrast, unmanned satellites can now be built at relatively lower cost, taking advantage of the improved reliability of modern electronic circuits, even those manufactured commercially. Indeed, the use of the Space Shuttle, a manned transporting system, as well as Spacelab, a manned facility, has in fact inhibited developments that would have led to lower costs. Experiments flown on Spacelab, if they had flown on unmanned satellites, could have cost a fraction of what was paid for them.

But unmanned spacecraft can be launched by Ariane, the European launcher, the development of which would

the Space Station, which will require the exclusive use of the Space Shuttle.

Moreover, European participation in the US Space Station would require European industry to use the expensive electronic parts made in the United States. As for the major part of the job, the life support system, European companies would have to build their share under American licences. The chief developments for European industry in such a participation could well be the production of screws and the bending of sheet-metal into interesting shapes.

In contrast, the development of unmanned space stations would force Europe to become more independent from the United States and could bring about the development of important technologies that are at present not available in Europe, perhaps not anywhere. This does not mean that joint efforts with the United States should not be sought. In the past, they have been fruitful, in many cases. But we should not move towards such cooperation on a space station.

There is no problem in constructing spacecraft in modular principles so that subsystems can be removed from the outside. Such repair can be performed by robots as easily as by men in space. Indeed, robots would be able to perform such repairs in any possible orbit — polar, eccentric or geostationary. There is no argument in favour of manned operations from the "repair" point of view.

Indeed, a recent study at Massachusetts Institute of Technology has recognized that robots, controlled according to anthropomorphic principles, may be the most promising route to future space techniques. This development was started 20 years ago when K. Kleinwächter in West Germany built such a system (called "Syntelmann"). There would have to be commodious communications links to send commands to a robot in space, supposedly to perform some operation. The robot may, for example, have a simulated arm. On the ground, a person would do the same experiment, when sensors monitoring the motion of the person's arm would relay appropriate commands to the robot and cause it to do exactly the same thing: opening a box, moving something, turning it. Such devices are also required on the ground, for operations in toxic environments, for example.

Opportunity

With this starting-point it would be tempting to develop, for example, unmanned spacecraft to support zero-gravity research. All the investigations required at this stage of the research could be accomplished in such a way. It would be necessary to develop sensors to generate images in all parts of the electromagnetic spectrum, from X rays to the far infrared. So as not to increase bit rates necessary in

such as on-board image evaluation, pattern recognition, redundancy reduction in information and the improvement of error detection and error-correcting codes for information transfer would be needed. If Europe, recognizing the enormous potential generally of such developments, were to move into such a direction, this would not only stimulate a broad field of scientific research (including for example, artificial intelligence by the application of the concept of associate memories) but would also require from industry products of high technological standard. The result would be to push European science and technology far ahead of where they are now — far beyond what participation in a space station programme might yield.

So why should Europe move along the dead-end route of the US Space Station? All experts agree that it would indeed be a dead-end. It is true that the idea has generated much science fiction — colonies in orbit, on the Moon or on other planets. But there is no possibility that we will be able to live on any other planet in the Solar System, or on an asteroid or on the Moon. There is no air to breathe while gravity is less than on the Earth, with possible consequences for human physiology. Neither the Moon, nor Venus or Mars have strong magnetic fields, so that their surfaces are bombarded by the full intensity of the cosmic radiation. The Moon has no atmosphere, that of Mars is dilute, so ultraviolet radiation and X-rays penetrate to the ground at high intensity. These two environments are a terrible threat to life. Venus has dense layers of ice clouds (from sulphuric acid) in its atmosphere, and almost 100 bar pressure at the surface, so it is dark and hot there; life would require permanent protection. Mercury is also extremely hot, and the outer planets are dark, as on Earth after sunset. So the dreams of life on other planets are unreal, pure fiction. We will stay on this planet. Why not accept that?

Is it not possible for a continent with the tradition of Europe, with its ability and with more than 300 million people, to stand on its own? Why not break with the traditional policy of imitating what is being done in the United States and, instead, move jointly in a promising direction which has been chosen deliberately by argument, not just by dreams or fears? If political unification is difficult in Europe, it should at least be possible to pursue common technical interests. Why not start with a powerful European space programme, designed to make good Europe's technological backlog, that will eventually generate contributions of general importance to our technical and technological development? Participation in the space station holds no such promise. So let us not participate. □

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Recollection of a simpler time

The death of Paul Dirac a week ago is a reminder of the precocious and staggering achievement, sixty years ago, of one of the most diffident of contemporary heroes

THE death of P.A.M. Dirac, in Tallahassee, Florida, on 20 October is a moving reminder of what science was like when everything was much simpler than now. The tale is that, as a shy but clever son of a Swiss-born schoolteacher in Bristol in the West of England, he embarked on a degree course in electrical engineering at the local university, just two months before the First World War ended. With a degree but no job, he took a degree in mathematics, as it were to fill in time. By 1923, with the help of a scholarship and a government grant, he was a research student at the University of Cambridge. And then, two years later, at the age of 23, he had put Heisenberg's quantum mechanics, not then published, on an axiomatic foundation that still endures.

How could so much have been done in so short a time? Luck must be a part of any explanation. Dirac was lucky that his Cambridge supervisor was R.H. Fowler, then full of excitement about the statistical mechanics of stellar atmospheres. Dirac was also lucky that Heisenberg went to Cambridge to talk at the Kapitza Club (then Cambridge's private *phi beta kappa*) in July 1925, and sent Fowler a set of the proofs of his first paper on new quantum theory by the middle of August. Who to ask to read them but Dirac? The real luck, of course, was that the time was ripe for a radical upheaval. Planck quanta were real enough but the old quantum theory of atoms, which worked well enough for atomic hydrogen, was increasingly at odds with spectroscopy.

But Dirac's special contribution to quantum mechanics was its independence of what others were about. Heisenberg's first paper (*Zeits.f. Phys.* 33, 879-893; 1925), the proofs of which Dirac had seen, had set the agenda — how to generalize Bohr's prescription that quantum states are those calculated by classical mechanics which happen to satisfy a quantum condition pulled like a rabbit from a hat. Heisenberg's innovation was to describe the kinematics and the dynamics of quantum variables strictly analogous to the classical dynamical variables — position, momentum and so on.

Dirac's first paper on the subject was received at the Royal Society on 7 November 1925, and published the following month in *Proc. Roy. Soc. A* 109, 642-653 (which says something else about the simplicity of those times). Its

It emphasized that the quantities representing dynamical variables in quantum mechanics are elements in an algebra then undefined, it demonstrated that the circumstance that two variables, say q (for position) and p (for conjugate momentum), do not commute is not an embarrassment (as it had seemed to Heisenberg) but a natural way of making a bridge with the quantities in classical Hamiltonian mechanics called Poisson brackets.

Dirac's achievement in the following five years was staggering. By the time his PhD thesis had been submitted (in the spring of 1926), he had made a first attempt at the problem of relativistic quantum mechanics. This was the train of argument that led to Dirac's relativistic equation of the electron which provided both a natural, not *ad hoc*, way of accommodating electron spin and as clear a prediction that positrons must also exist as any more recent prediction of a particle of matter. Almost in passing, Dirac quantized Maxwell's equations, providing the foundations of a quantum theory of radiation and a model for what is now called field theory.

Throughout this revolution, Dirac's style was distinctive. He was not so much an iconoclast as an adventurous spirit, sceptical of fudges but prepared to see where logic would lead. What else can account for his failure to accept as his own the idiom in which his elders and presumptive betters described the problems facing physics in 1925?

The point is nicely illustrated by Dirac's own algebra for the dynamical variables of a quantum system. Heisenberg had shown that the quantum condition boils down to the simple relation that $qp - pq = i\hbar$ if q and p are, respectively a position and its conjugate momentum (with i the square root of -1 and $\hbar$ Planck's constant divided by 2π). When Born and Jordan had reminded Heisenberg that his quantum equations embodied the rules of matrix multiplication, the three went on to elaborate their doctrine of matrix mechanics.

Not Dirac. The quantum variables were the elements of an algebra whose rules were dictated by the physics. Dirac called them q -numbers (*Proc. Roy. Soc. A* 110, 561-569; 1926) and said this about them:

At present one can form no picture of what a q -number is like. One cannot say that one q -number is greater or less than

that if z_1 and z_2 are two q -numbers... there exist the numbers $z_1 + z_2$, $z_1 z_2$ and $z_2 z_1$ which will in general be q -numbers... One knows nothing about the way the numbers are formed except that they satisfy all the ordinary laws of algebra, excluding the commutative law of multiplication.

At any point along the way, Dirac could have resolved this uncertainty by changing idiom. Representing q -numbers by matrices would have given Heisenberg's quantum mechanics, representing them by differential operators would have given Schrödinger's wave mechanics. But why needlessly particularize?

The scale of Dirac's achievement during this short spell is now not easily comprehended. It is as if he had found a secret window on the foundations of physics, a companion for that through which Einstein looked in 1905. It cannot often have been that a physics textbook (*The Principles of Quantum Mechanics*, Oxford, 1930) should be nothing but a record of its author's research.

Dirac's distinctive style stamps this great book and the generation of students brought up on it — or in its shadow. Dirac himself, however strongly convinced in 1930 that the problems of physics had been solved, seems not to have been filled with proselytizing zeal. Always shy, he seems to have been tragically shy of students. Much of the 1930s were spent in a hunt for some way of ridding calculations of radiation fields of the infinities that bedevilled them. Dirac did not succeed in this herculean task, completed only in 1948 by Schwinger, Tomonaga and Feynmann.

But from this period there survive three novel ideas — the notions that magnetic monopoles may exist, that it cannot be a mere coincidence that dimensionless numbers such as the ratio of electrical to gravitational forces are equal to the age of the Universe (in atomic units) and (as a consequence) that the gravitational constant should decrease with increased age of the Universe (*Nature* 139, 323; 1937). Time, no doubt, will show what happens to these conjectures.

Dirac's style persists. The anonymous reviewer of the first edition of his book (*Nature* 127, 699; 1931) said of Dirac that "he bids us throw aside preconceived ideas regarding the nature of phenomena... we may describe this as the application of pure thought to physics". He was a subversive whose pure thought was more penetrating

Molecular medicine

Therapy by instant evolution

from Robin Carrell

EVOLUTION has modified and refined the structure and function of proteins. An understanding of how this has happened makes feasible the prospect of artificially inducing similar changes to create specifically designed variants of human proteins. As a starter, on page 77 of this issue¹, Rosenberg *et al.* (Chiron Research Laboratories) report the use of recombinant DNA technology and yeast to replace the amino acid at the reactive centre of human α_1 -antitrypsin, a natural inhibitor of protease enzymes. The mutant α_1 -antitrypsin retains the specificity of the natural inhibitor, but promises to provide more effective protection to tissues during inflammatory stress. Of particular interest is its potential to prevent the development of the degenerative lung disease, emphysema, but the independent production of related mutants of α_1 -antitrypsin by two other genetic engineering companies, Transgene, Strasbourg (M.A. Courtney *et al.* *Nature*, in the press) and Biogen, Cambridge, Massachusetts (D. Schindler, personal communication) attests to much wider therapeutic implications.

This focus of activity, follows from the realization² that α_1 -antitrypsin is one of a family of serine protease inhibitors, the serpins, each of whose function is critically determined by a single amino acid at the reactive centre. These protease inhibitors have specific roles in the control of the proteolytic cascades responsible for immune reactions, for coagulation, and for other responses to inflammation. Since it is the aberrant activation of these cascades that underlies many disease processes, there is great medical interest in the possibility of specific intervention by the use of engineered inhibitors.

α_1 -Antitrypsin is the archetype³ of the serpin family which includes the plasma proteins: antithrombin, α_1 -antichymotrypsin, C₁-inhibitor and α_2 -antiplasmin⁴. The structural homologies of the family, together with recent crystallographic studies⁵ of α_1 -antitrypsin, indicate that the serpins all share the same highly-ordered globular structure. A feature of this structure is the exposure of the reactive centre as a loop on the exterior of the molecule. This loop is believed to fix the reactive centre in a conformation that makes it an ideal substrate for the target protease. The inhibitor and its target protease thus form a tight 1:1 complex which can be rapidly recognized and removed from the circulation.

The specificity of each inhibitor is critically dependent on a putative cleavage site at the P₁ residue (see the figure) of the reactive centre of the inhibitor. This was

tive centre of α_1 -antitrypsin, which has methionine at P₁ and is primarily an inhibitor of elastase, with that of antithrombin, an inhibitor of thrombin which has arginine at P₁. Unequivocal evidence that the P₁ residue was responsible for the specificity came from an unusual experiment of nature⁶: a child from Pittsburgh was found to have a bleeding disorder associated with a new genetic variant of α_1 -

Serpin	P ₂	P ₁	P ₁ '	P ₂ '	P ₃ '	P ₄ '	Target
α_1 -Antitrypsin	Pro-	MET	-Ser-	Ile-	Pro-	Pro-	Elastase
Valine mutant	Pro-	VAL	-Ser-	Ile-	Pro-	Pro-	Elastase
Pittsburgh mutant	Pro-	ARG	-Ser-	Ile-	Pro-	Pro-	Thrombin
Antithrombin	Gly-	ARG	-Ser-	Leu-	Asn-	Pro-	Thrombin
α_1 -Antichymotrypsin	Leu-	LEU	-Ser-	Ala-	Leu-	Val	Chymase
Mouse contrapsin	Arg-	LYS	-Ala-	Ile-	Leu-	Pro	'Trypsin'
C ₁ -inhibitor	Ala-	ARG	-Thr-	Leu-	Leu-	Val	Kallikrein

Reactive site sequences of natural human serine proteinase inhibitors (serpins) and two mutants of α_1 -antitrypsin. Cleavage putatively occurs at the P₁-P₁' bond with a specificity of inhibition that is clearly related to the P₁ residue (C₁-inhibitor sequence is a personal communication from J. Travis).

antitrypsin, in which the reactive centre methionine had been replaced by an arginine; as predicted, the mutant α_1 -antitrypsin had lost the ability to inhibit elastase but had become a highly effective inhibitor of thrombin.

The general applicability of this dependence on the P₁ residue was recently demonstrated by Hill and colleagues⁷, from a comparison of the amino acid sequences deduced from the cDNA of mouse and human serpins. Their most remarkable conclusion concerned human α_1 -antichymotrypsinogen and mouse contrapsin. These two proteins have a similar sequence but the human inhibitor has a leucine at P₁ and hence inhibits chymotryptic-like proteases, whereas the mouse inhibitor, with a P₁ lysine, has no antichymotryptic activity but predictably is as an efficient inhibitor of trypsin.

Evolution, in several experiments of her own, has thus demonstrated how to prepare specific inhibitors. Rosenberg *et al.*¹ have applied these lessons to the production of a mutant α_1 -antitrypsin in which the P₁ methionine has been replaced by a valine. Their choice follows from the fact that about one in a thousand northern Europeans has a severe genetic deficiency of α_1 -antitrypsin and is consequently susceptible to cumulative damage to the elastic tissue of the lungs, which results in emphysema. In principle, the defences of susceptible individuals could be improved by intermittent transfusions of α_1 -antitrypsin. Genetic engineers, anticipating this need, have taken the opportunity to produce a modified form of

and so provide improved protection.

The risk of damage to the lungs in both normal individuals and those deficient in α_1 -antitrypsin is heightened by the susceptibility of α_1 -antitrypsin to inactivation by oxidants released by leukocytes or present in cigarette smoke⁴. There is strong evidence that this is due to the oxidation of the P₁ methionine to methionine sulphoxide, with consequent loss of effectiveness as an inhibitor of leukocyte elastase. Improved protection might be provided by the replacement of the P₁ methionine of α_1 -antitrypsin with a residue resistant to oxidation^{3,4} — valine seems a logical choice⁸ for an inhibitor of leukocyte elastase. *In vitro* tests of the mutant that fits that bill support those predictions. Before the material is ready for therapeutic use, several questions will need an answer. Does it matter that the mutant protein, as produced by yeast, does not have oligosaccharide sidechains? Antigenicity will probably not be a problem, but how important is it that the mutant inhibitor is likely to be less soluble and long-lived than the natural form? This will be more of a problem if the material is to be administered by transfusions than if it can be directed to the lungs as an aerosol. A greater problem will lie in the assessment and marketing of an agent whose protective effect, with regards to emphysema, is measurable over years rather than days.

Other related mutants that are at the testing stage may have more immediate medical applications. It can be predicted that the P₁ arginine mutant^{1,6} will be an effective medium-term anticoagulant for individuals with either a genetic or functional deficiency of antithrombin. Here, a shortened half-life due to lack of glycosylation may prove an advantage by increasing precision in the day-by-day control of anticoagulation.

In a wider sense, the new papers underline the rapid growth in the practical applications of molecular biology. When we suggested the possible production of the valine α_1 -antitrypsin³ it was done so almost tongue-in-cheek. Yet 18 months later it has been achieved. The speed with which molecular biology is growing has turned protein chemistry into an adventure rather than threatening it, and has converted the academic exercises of correlating protein structure with function into matters of direct, practical importance. □

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Oceanography

Tides, solitons and nutrients

from Melbourne G. Briscoe

TIDAL energy mixes the coastal waters, providing nutrients for biological processes. The mechanism is indirect; the topography of the shelf-break, the stratification of the coastal waters and the tides interact to produce groups of solitons — strong, short, high frequency internal waves — and it is the breaking of these solitons as they propagate inshore to even shallower water that is responsible for the mixing. That is the suggestion offered by H. Sandstrom and J.A. Elliott of the Bedford Institute of Oceanography in Canada¹. Their idea, based on their work on the Scotian Shelf, seems sound but perhaps does not go far enough.

Solitons are so-called because of their similarity to the mathematically precise idea of a non-linear wave that retains its identity and form as it propagates along as a singular bump on the sea surface. However, in most situations an internal wave actually travels as a singular wave of depression, since there is usually a deeper layer of water underneath the pycnocline (the layer where the water density changes most quickly with increasing depth) than above it. As the internal depression on the pycnocline propagates, it tends to disperse into the free-wave components that compose it. This tendency is balanced by the non-linearities that arise from the finite amplitude of the depression and allow the wave to retain its soliton form. This fragile balance of forces is easily realized in equations, can sometimes be simulated in the laboratory, but probably never really occurs in the ocean; I therefore use the word soliton with some reservations.

Solitons are thought to be produced by the surface tide running up against the edge of the continental shelf. The pycnocline overlying the shelf is disturbed vertically by the tidal flows and a depression in the pycnocline begins to travel shoreward. There is some uncertainty about the mechanism that forms the depression: Sandstrom and Elliott consider that the surface tide forms an essentially linear, internal tide at the shelf edge, and that the leading edge of the propagating internal tide is the depression that finally resolves into the soliton¹. The scales of these processes differ greatly. The surface and internal tides (called barotropic and baroclinic tides, respectively, in the oceanographic literature) occur once or twice a day, depending on location, but the wave sequence in the soliton is of just a few minutes period. The horizontal scales of these three processes decrease from thousands of kilometres, to tens of kilometres, to hundreds of metres.

Because of the tidal generation of solitons they can be recorded every 12.4 h in those locations where the semi-diurnal tide

is dominant. One might also expect a spring-neap cycle in the intensities of the process, but I am aware of no good data that confirm this; since the dynamics of the generation of the solitons and of their eventual propagation depend on the strength and depth of the pycnocline, the inevitable changes in the hydrography with space and time probably obscure spring-neap effects.

Surprisingly, solitons are often easy to observe in the ocean. The interaction of wave currents in the soliton with short surface wind waves causes a modulation of the roughness of the sea surface that is visible to the eye and to radar. Landsat satellite images described by C. Sawyer² show that the entire eastern seaboard of North America is covered with a pattern of surface streaks characteristic of solitons. This is especially so in the 50–150 m depth range of the shelf, and is most marked in summer and early autumn, when the thermocline is well developed. (Georges Bank and its surroundings perhaps have the most solitons in the western North Atlantic; does this account for the nutrient supply and the fishing industry on Georges Bank?)

Sandstrom and Elliott propose that the surface tides at the shelf edge are converted to internal tides and then to solitons that dissipate on the shelf¹. They calculate a figure of 6×10^7 J per metre of crest length for their solitons. If extrapolated to the coastlines of the world this yields 1×10^{15} J, less than a third of a percent of the total internal tidal energy, a negligible contribution to the problem of how the energy is

dissipated. On the other hand 6×10^7 J dissipated on a 10 km wide shelf in half a day provides about 50 mW m^{-2} towards the vertical mixing of the water and the erosion of the base of the mixed layer. This is at least ten times the energy supply estimated from internal wave dissipation in the deep sea and several times larger than estimates of the energy available from typical winds.

A.R. Osborne and T.L. Burch³ have described solitons in the Andaman Sea near Sumatra that are 15 times more energetic than the examples of Sandstrom and Elliott. Even if all of the coastlines of the world were covered with such solitons, and all these solitons were dissipated in half a day, this would still only account for 0.3×10^{12} W, just 20 percent of what needs to be dissipated from the surface tide in shallow seas according to G. Miller's calculations⁴. (These take into account surface tide dissipation due to conversion to internal tides or solitons on the shelves for some areas of the world, but not for the North American east coast where so many solitons have been observed.)

So even this extreme estimate of the energetics and dissipation of solitons, we are not much closer to understanding where tidal energy goes, but Sandstrom and Elliott's suggestion¹ of the importance of solitons for shelf-mixing and for the supply of nutrients to the euphotic zone seems plausible on the Scotian Shelf. It may even be applicable to thousands of kilometres of other soliton-covered shelves. □

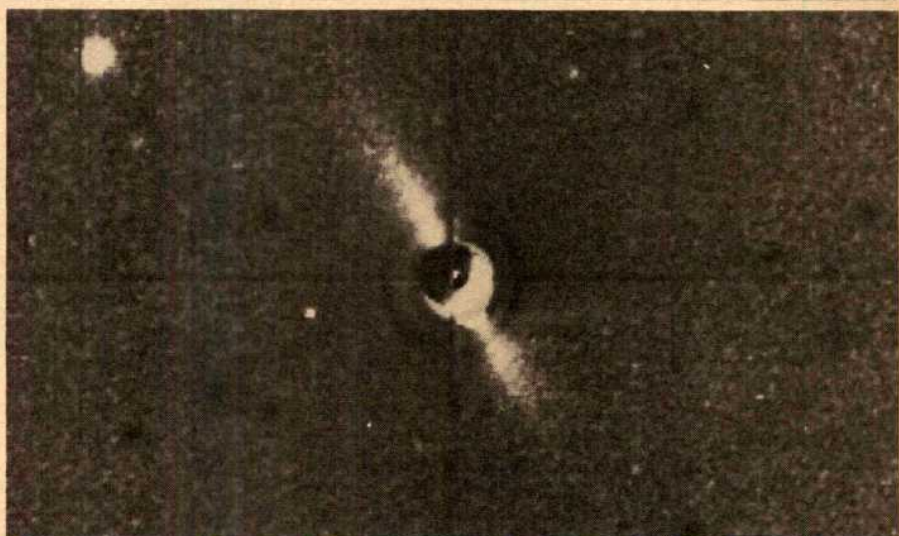
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A masked image of the star β Pictoris taken by R.J. Terrell (Jet Propulsion Laboratory) and B.A. Smith (University of Arizona) using a charge coupled device on the 2.5 m telescope at Las Campanas Observatory, Chile. The bright extended emission is thought to come from a circumstellar disk of silicates, ices and carbonaceous organic material. The disk, seen edge-on, is 40 billion miles across; the star is 50 light years away. This disk is the first of its kind to be imaged directly.

T-cell antigen receptor

The capture of the snark

from Miranda Robertson

WHEN I last discussed antigen recognition by T cells five weeks ago¹, I confined myself to what could be learned from the genes encoding the beta chain of the receptor heterodimer, on the grounds that there was serious doubt about the authenticity of the putative alpha-chain gene isolated by Saito *et al.*². There is no longer any doubt.

In this issue Hannum *et al.*³ report amino-acid sequences from a human T-cell receptor alpha chain that clearly have no homology with the amino-acid sequence predicted from the mouse gene of Saito *et al.*²; moreover, the alpha-chain protein is glycosylated whereas the predicted product of the gene has no glycosylation sites. And at the same time, Chien *et al.*⁴ and Saito *et al.*⁵ announce the isolation from mouse T cells of what both cautiously describe as a third type of T-cell receptor gene: in fact, thanks to the protein sequence of Hannum *et al.*, these genes can be unambiguously identified as alpha-chain genes. Yet the original Saito *et al.* gene², which I shall call the non-alpha gene, has all the important properties expected of a T-cell receptor gene; which leaves us, apparently, with one more receptor gene than the protein data had led us to believe we needed.

Since this surprising finding has given rise to speculation that antigen recognition in T cells may require two receptors, I shall briefly rehearse the arguments for and against such a schema in the context of what is now known about the expression of the alpha- and beta-chain genes, before going on to discuss a paper that contains a detailed analysis of several beta-chain genes and may bear on the alternative possibility of dual recognition by one receptor⁶.

How many receptors?

The notion that T cells may require two antigen receptors originated with the discovery that, unlike B cells, which recognize antigen on its own, T cells recognize antigen only in association with cell surface molecules encoded by the major histocompatibility complex (MHC). Specifically, cytotoxic T cells recognize antigen in association with class I MHC molecules whereas the regulatory T cells — the helper and suppressor cells — respond to antigen in the context of class II MHC molecules. Hence the suggestion that T cells might express two receptors, one for antigen and the other for the products of the MHC.

Under such a two-receptor scheme, the non-alpha gene might encode the MHC receptor. Its relative invariance, which was one of the strongest arguments against it as the alpha-chain gene, would be somewhat less of a handicap to a gene encoding a receptor for MHC molecules. Even so

MHC polymorphism might demand rather more than the junctional diversity generated during the assembly of the separate gene segments encoding the variable (V) region of the receptor (for fuller explanation see ref. 1).

There are, however, more serious difficulties with this interpretation of the non-alpha gene. First, immunoprecipitation of the T-cell receptor brings down only the alpha and beta chains, together with the so-called T3 marker molecule, which is coordinately expressed on all T cells with the alpha and beta chains⁷ and whose major chain, unlike the non-alpha chain, has no homology with immunoglobulins⁸. Second, Chien *et al.*⁴ report that the non-alpha chain is expressed at much lower levels in T cells of thymus and spleen than the alpha- and beta-chain genes. Most serious of all, there is compelling evidence from cellular immunology^{9,10} that a single receptor is responsible for the dual recognition of antigen and MHC molecules.

It remains possible that the non-alpha chain exists as a minor isotype, analogous to the lambda light chain of immunoglobulin — which is also relatively invariant. In that case, however, it ought not to be expressed along with both the other chains in the cytotoxic cell line of Saito *et al.* (B cells expressing lambda light chains do not also express kappa chains). There is, in fact, so far no evidence that the non-alpha product is actually made and expressed on the surface of the cell, and since there are numerous precedents for aberrant transcripts the isotype hypothesis cannot be ruled out. On the other hand, until there is evidence that the non-alpha gene is expressed on the surface of one cell or another, its status as a functional gene must be considered unproven.

How like immunoglobulin?

How then is dual recognition reflected in the structure of the alpha-beta heterodimer? Before considering the structure of the beta-chain V regions, I must first dispose of the question of whether the differential expression of different alpha- and beta-chain gene pools could account for the differential recognition of class I and class II molecules by cytotoxic and regulatory T cells. I have already quoted¹ the evidence that the same beta-chain gene pool is expressed in cytotoxic and helper T cells, and it is now clear that both also express the same alpha-chain genes: the constant-region (C) segment of the alpha cDNA clone isolated by Chien *et al.* from a mouse helper cell is identical to that of the alpha-chain clone isolated by Saito *et al.* from a mouse cytotoxic cell. However, the variable regions are clearly very different

which is to be expected in the light of the evidence for V-region diversity in the alpha-chain protein^{11,12}.

This brings us to the question of how the V regions of the alpha and beta chains might provide a structural basis for dual recognition. The homologies, both in sequence and in organization, between immunoglobulin and T-cell receptor genes, have already been discussed in *News and Views*^{1,13}. Because immunoglobulin recognizes only antigen, however, while the T-cell receptor recognizes both antigen and MHC, one would also expect profound differences. Such differences, chiefly in the pattern of diversity of the V regions, are now reported by Patten *et al.*⁶.

I have already mentioned¹ two ways in which beta-chain V-region diversity seems to differ from that of immunoglobulins: first, there is so far no evidence for somatic mutation in beta-chain genes; and second, there seems to be more scope for diversification during the assembly of the three gene segments encoding the variable region, in that it is possible in principle to assemble V genes either with no D segment or with more than one (for details and explanatory diagrams see ref. 1). What Patten *et al.* now show is that any such mechanisms for increasing somatic diversity of the receptor genes are acting on an already very heterogeneous set of germline V segments.

They have analysed beta-chain V sequences from seven helper cells and find that, unlike the immunoglobulin V segments, which tend to run to family sizes of up to 30, V_β gene families seem not to exceed two or three sequences. Moreover, there is very low homology between families: the least homologous immunoglobulin V segments are about as similar as the most homologous V_β sequences. More detailed analysis of this diversity has shown that it can be attributed to seven hypervariable regions, three equivalent to the classical hypervariable regions of immunoglobulin, two more which also have counterparts in immunoglobulin, and two that are exclusive to the T-cell receptor. From predictions of secondary structure based on primary sequence data, and on the assumption that the tertiary structure will be similar to that of immunoglobulin, Patten *et al.* conclude that the three classical hypervariable regions would, like those of immunoglobulin, form the antigen-binding pocket of the molecule, but giving a deeper cleft; while the remaining hypervariable regions would fall on the exterior of the folded protein and might mediate interactions with MHC molecules.

They further speculate that MHC binding might induce allosteric changes in the antigen-binding pocket that would represent a further source of diversity in the range of binding combinations possible. Some very preliminary unpublished evidence is quoted by Patten *et al.* in support of such allosteric interactions: two T cells recognizing the same antigen in association

with different MHC molecules express different V-region genes.

What next?

At this stage, there are not enough data to justify detailed speculation on the mechanism of dual recognition. If the non-alpha chain plays a part in the recognition of MHC molecules, then one might expect that, unlike the alpha- and beta-chain genes, it would be differentially expressed (if it is expressed at all) in different subsets of T cells. Tonegawa has preliminary evidence suggesting that it may indeed be transcribed only in cytotoxic and not in helper cells. In that case, one would also predict an equivalent non-alpha, non-beta chain mediating the recognition of class II MHC molecules in the regulatory subsets.

With all the relevant genes now isolated — both the genes encoding the MHC mole-

cules that the T-cell receptors recognize, and the genes encoding the receptor that does the recognizing — it should be possible to devise transfection experiments that will answer the questions raised in this article. □

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Space missions

Very-long-baseline interferometry takes to the sky

from P.N. Wilkinson

RADIO interferometers have long since surpassed the best angular resolution achievable with ground-based optical telescopes but radio astronomers have continued to strive for higher resolution in the quest of new information about cosmic objects. Since 1967, when the technique of very-long-baseline interferometry (VLBI) was introduced, we have been aware that there is a natural limit to this process — the separation of our telescopes is restricted by the size of the Earth itself. Nonetheless, there have so far been enough scientific rewards from exploiting existing radio telescopes for us not to worry too much about limited resolution.

Now, however, in common with many other astronomers, our thoughts are moving beyond the Earth towards an observatory in space, QUASAT (for QUASar SATellite), which would be linked to the earthbound network of radio telescopes. The initial ideas for this project were discussed recently at a workshop* sponsored by the European Space Agency (ESA) and the National Aeronautics and Space Administration (NASA), attended by astronomers, engineers and administrators from twelve nations.

Much of the present interest in VLBI stems from the recent extension of the principle of 'aperture synthesis' to arrays that span intercontinental distances; as a result, radio maps with milli- and sub-milliarc second resolution can now be made routinely. But many extragalactic sources and components in water-line masers are barely resolved even on the longest Earth baselines and at the shortest wavelength (1.35 cm, a frequency of 22

GHz) at which most large radio telescopes work well. Higher resolution, at least on continuum sources, is achievable at millimetre wavelengths but millimetre-wave VLBI is only in its infancy. To take advantage of the experience at centimetre wavelengths and to use the more numerous and much larger centimetre wave telescopes, the physical length of the baselines must be extended. This can be done only with the aid of a radio telescope in space.

QUASAT's astrophysical importance depends on its sensitivity, and on the resolution and quality of the synthesized image. In this 'first step' mission compromises must be made between these criteria. Thus the present proposal, which is dominated by the desire to observe at 22 GHz, has a relatively small antenna and hence sensitivity at the lower frequencies has been conceded for resolution. Nonetheless, QUASAT would have plenty to look at in its up to 5 year lifetime. The proposed orbit would yield roughly a three-fold better linear resolution, or a 10-fold smaller pixel area in the resulting image than Earth-based arrays. At 22 GHz the resolution is about 100 μ arc s, corresponding to a size scale ranging from 1 AU (the distance from the earth to the Sun) at the distance of the galactic centre (10 kpc) to about 10,000 AU at a distance of 100 Mpc. Computer simulations presented by A. Readhead (Caltech) show that with QUASAT and typical 10-station ground arrays, it would be possible to make radio images of a quality similar to that now achieved with multiple 'snapshot' observations using the Very Large Array (VLA), but with almost 1,000 times higher resolution; and that VLBI work in the

lutionized. Furthermore, using a worldwide network of 34 ground observatories which could be available in the 1990s, it would be possible to produce radio images of the compact nuclei of active galaxies as complex as any that the VLA has so far produced.

There was no shortage of exciting ideas for what the QUASAT mission could achieve. Superluminal sources — in which there are ordered motions with apparently faster-than-light speeds — are among the obvious targets. Even after a decade of observations, we have no real physical understanding of these phenomena. A closely related goal is to study the propagation of the energy-carrying beams, which emanate from the 'central engine' in active galactic nuclei, as they pass through the region that is responsible for producing the broad optical emission lines. We now know that in the much larger narrow-line region, the line-emitting gas clouds can bend and brake the beams; thus the interaction probes the physical conditions both in the clouds and in the beams. QUASAT will extend this study to the broad-line region, which is inferred to be less than a light year across in many cases, and which therefore cannot be studied directly even with the Space Telescope.

On the smallest accessible scales QUASAT could test the long-standing prediction that the brightness temperature of incoherent synchrotron emission is limited to 10^{12} K by inverse Compton scattering. It cannot be tested using Earth baselines but the QUASAT baselines would record 10^{13} K emission if it exists. The super-bright emission could be from a previously unsuspected coherent radiation source or, more likely, it could indicate that the emission region is approaching us at relativistic speed. In this case the apparent brightness temperature in the observer's frame is increased because of the effects of aberration.

Several speakers stressed QUASAT's great potential for studying the physical conditions in hydroxyl (at 1.6 GHz) and water (at 22 GHz) masers which are associated with star-forming regions and some late-type stars. Perhaps the most exciting prospect, however, is to use the water-masers to make fundamental distance measurements. The internal motions of water-maser spots, already revealed by Earth-based VLBI, have enabled the distances of three galactic maser complexes to be measured by statistical parallax techniques (Moran, J.M. *Nature* **310**, 270; 1984). QUASAT would greatly facilitate these measurements and would allow the distances to many more star-forming regions to be determined. And M.J. Reid (CFA) introduced the even more tantalizing prospect of using masers to measure the distances of nearby galaxies, thus helping to clarify the contentious extragalactic distance scale. Powerful

galaxies (Moran, *op. cit.* and Claussen, M.J. *et al. Nature* 310, 298; 1984) and we could use the statistical parallax technique if we could measure their internal motions with an accuracy of a few microarc seconds per year.

With QUASAT, it might also be possible to determine the orbital motions of maser sources around a galaxy. These angular motions (corresponding to velocities of a few hundred km s^{-1}) will be an order of magnitude larger than the internal motions in the masers themselves. If the rotation curve and the inclination of the galaxy are known, then a distance estimate can be derived directly.

One debate at the workshop centred on the scientific trade-offs between raw resolution and imaging capability. A vocal minority took the view that much cruder images, but with two or three times greater resolution, might lead to more fundamental discoveries. Where to draw the line between resolution and image quality is a matter of judgement but clearly more computer simulations are needed to investigate the point at which image quality deteriorates to levels unacceptable to all.

R.Z. Sagdeev (Space Research Institute, Moscow) gave a brief, but up-to-the-minute account of current Soviet thinking. The USSR hopes to place a large (30 m) antenna in low Earth orbit by the end of this decade. This spacecraft, working with ground telescopes in the USSR, would produce a well-filled aperture but with maximum baselines little longer than can be obtained with current VLBI networks. Thus it is being regarded as a step towards a more ambitious mission which might, like QUASAT, be launched in the 1990s. This second mission resembles QUASAT in several ways: for example, it would employ wider bandwidths and higher observing frequencies. It also offers an appealing way around the trade-off between resolution and image quality which inevitably arise when only one orbiting telescope is used. There would be much to be gained if QUASAT and the Soviet telescope (unofficially dubbed KBACAT) were to be placed in complementary orbits. In the most entertaining few minutes of the entire meeting J.F. Jordan (Jet Propulsion Laboratory) showed a time-lapse movie simulation which demonstrated graphically how, in this regard, the QUASAT-KBACAT baselines would enable us both to have our cake and to eat it, for both longer baselines and a better filled aperture plan are then possible.

QUASAT seems destined to be a competitor in the 1987 selection round in ESA and could get a 'new start' in NASA that same year. The antenna concept is expected to be selected in summer 1985, with a joint NASA-ESA phase A study in 1986. Launch could be in 1992. □

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Martin Ryle 1918-1984

SIR Martin Ryle, pioneer of radio astronomy and explorer of the Universe, died on 14 October 1984. His insight and originality in radio science was first displayed in wartime, when he was recruited directly from Oxford in 1939 to work in the Telecommunications Research Establishment. Here his first tasks were to develop airborne radar aerial systems and to test equipment for the new centimetric waveband; these provided an introduction to radio techniques which prepared him well for his vital, but almost unrecorded, contributions to radio countermeasures.

It was Jack Ratcliffe who persuaded Ryle to start radio research in the Cavendish in 1945, with the support of an ICI Fellowship. The wartime discoveries by John Hey, of intense radio emissions from the Sun and from a mysterious source in the constellation of Cygnus, provided challenging subjects, well suited to Ryle's experience in the location of radio transmitters and the recognition of unusual signal characteristics. He devised a radio interferometer, analogous to Michelson's optical counterpart and soon named after it, which showed in 1946 that sunspots emit powerful, circularly polarized radio waves, and that the quiet sun is surrounded by a hot, radio-emitting corona. A succession of students was encouraged to map this coronal emission, using ingenious developments of the interferometer; these developments can now be seen as the first stages of the technique of aperture synthesis, one of Ryle's most famous achievements.

The investigation of the Cygnus radio source followed similar lines. It was a natural hypothesis that this source might be a star that radiated like the Sun but much more powerfully. The successful location of the source, and its identification as a very distant and very unusual galaxy, opened the way for radio astronomy to investigate the structure of the Universe on the largest scale. At that time, there could be no clear interpretation of the many discrete radio sources appearing in the early surveys; they were at first supposed to belong to our Galaxy, where they would account for the background radio emission from the Milky Way. Ryle determined to survey and locate a large number of these sources. For this purpose he built a large interferometer array, working at a wavelength of 3.7 m. The results were astonishing: the sky was apparently crowded with sources, with almost no concentration in the Milky Way.

In his 1955 Halley lecture, Ryle showed that most of these radio sources must be extragalactic and so distant that they gave the first clear evidence which could be used to distinguish between cosmological theories. His results and their interpretation were immediately the subject of

not agree with the number counts of the radio sources, while the theorists vigorously defended the sinking ship of the Steady State. The conflict could only be resolved through better observations, which Ryle duly provided by improving the interferometer in successive steps, leading eventually to aperture synthesis.

Aperture synthesis, the simulation of a very large radio telescope by the use of multiple interferometers, was Ryle's supreme invention. It was achieved in 1956, when the technical problems of computation were overcome by the use of the pioneering digital computer, EDSAC. Its full development progressed through the 'one-mile' telescope (1964), which used three 18 m paraboloids, giving an angular resolution better than one arc minute, to the well-known 'five kilometer' telescope (1972), working on shorter wavelengths: this telescope gave, for the first time, an angular resolution of one arc second, similar to that of optical telescopes.

The results, both in the form of number counts and as detailed maps of radio galaxies and quasars, firmly established radio astronomy as the main source of direct observational evidence in cosmology. There were, of course, many by-products, most notably Hewish's discovery of pulsars, which led to Ryle and Hewish sharing a Nobel prize in 1974.

Ryle's originality and determination marked the whole development of Cambridge radio astronomy. He was a magnetic figure, with the power of inspiring of a deep loyalty and a social cohesion among a long succession of students. He had a deep social conscience, which led him in his later years to an active and public concern about nuclear power and armaments. He will be remembered with affection by everyone who worked with him.

F. Graham Smith

100 years ago

EARTHQUAKE MEASUREMENT

IN an article on "Earthquakes" in last week's *Nature* (p. 608), Dr. H. J. Johnston-Lavis takes exception to the records of earthquake motion which I have published, on the ground of their complexity, and pronounces the Plain of Yedo unsuitable for earthquake observations.

Now this seems to me to be a very eclectic way of treating earthquakes. We can measure earthquakes only where we find them, and I suppose the first qualification in a site for an earthquake observatory is that there should be plenty of earthquakes. The Plain of Yedo possesses this qualification in a very high degree; and if the disturbances which occur in it are of a very much more complex character than our *a priori* notions about earthquakes may have led us to expect, it is not the Plain of Yedo that is to blame.

University College, Dundee.

J. A. Ewing

Anthropology

Prehistory of Amazonian Indians

from Robert M. May

TIME was when the popular image of the Amazonian rain forest was of a teeming paradise with great potential for sustained productivity. Following the collapse of many ill-conceived 'development' projects, however, the contemporary image is more one of fragile forests perched on impoverished soils, or even of a Green Hell¹. Consistent with this recent picture is a vision of the Amazonian Indians as simple people eking out a precarious existence, with population densities that have always been too low to sustain any complex social organization or culture. A series of recent studies contest this view of the Amazonian Indian²⁻⁶. Several studies argue that prehistoric population densities were an order of magnitude or more higher than conventional estimates, or than densities found today. Moreover, it is argued that the Indians' knowledge about and use of their environment was much more sophisticated than has usually been believed.

Population

The early estimate of the density of aboriginal Indians in Amazonia before the advent of Europeans was less than one individual per km². As summarized by Dobyns² in an important revisionist article, this estimate is based partly on historic records, partly on assessment of the carrying capacity of the environment and partly on direct observations of contemporary densities of aboriginals. Dobyns discusses each of these three approaches.

First, most historians and anthropologists have tended to discount contemporary sixteenth and seventeenth-century estimates of Indian populations in both North and South America as gross exaggerations. Another revisionist, Myers³, is understanding of such discounting, even though he disagrees with it: "we must recognize that the earliest historical sources were written either by adventurers in search of gold and kingdoms to conquer, or by missionaries whose vast expenditures could only be justified by a proportionate number of conversions to the Catholic faith. When disinterested parties finally did reach the Amazon, they found no evidence of the large populations which were reported to have lived there". Dobyns shows, however, that when the old records — or indirect inferences drawn from them — are examined carefully, and cross-checked one against another where possible, their high estimates of the initial size of aboriginal populations in North America, in Central America, in the Incan Empire and in Amazonia appear to stand up. Discrepancies arise because populations crashed between first contact and the arrival of

diseases introduced by Europeans. Likewise, Myers's detailed analysis of all available historical records for the Indians along the Ucayali River in eastern Peru indicates that "large and stable communities were in fact characteristic of the mainstream Ucayali tribes at the beginning of the historic period, but that they collapsed with the precipitous population declines caused by Spanish diseases".

Second, our understanding of the ability of the Amazonian rain forest to sustain humans is not good enough to provide any reliable estimate of population sizes (more on this below).

Third, direct observations of population sizes several years after European contact are not useful, by virtue of the havoc



Buhaqana Indian, East Colombia, c.1890. (Peter Newark's Western Americana).

wrought by disease. Such useful observations as do exist come from geographically isolated areas of the Amazon Basin where a few native populations appear to have experienced their first effective contact with Europeans so recently that ethnologists have been able to collect information about the population before contact. One group, first contacted in 1926, was virtually wiped out by influenza in 1929. In another, 300 died of pulmonary oedema in 38 hours. Another group of 300 Sabane came into a Brazilian post, where the wagon bringing gifts and rations for them also brought bronchial pneumonia; the few survivors fled back to their settlements, spreading the epidemic. Anecdotes of this kind make it clear that

decades after first contact give little hint of pristine population densities².

In pursuit of an estimate of the depopulation typically caused by disease, Dobyns pulls together information from various places in the New World and concludes² that over the 100 years following first contact, "the depopulation ratio of 20 to 1 appears to be a sound, if perhaps conservative, tool to employ as a hemispheric minimum". This figure coincides with an earlier and less encyclopedically-based estimate by Borah of a 95 per cent reduction by disease in native populations².

As Myers has emphasized elsewhere, viral and bacterial diseases can travel along trade routes, accompanying items of trade into remote regions long before the first face-to-face contact with Europeans is made⁴. Thus, the first reported contact may be with a native population already severely reduced by disease.

A different and independent indication that Amazonian Indian populations were relatively dense before the arrival of Europeans comes from analysis of sites of 'Indian Black Earth' (*terra preta do indio*)⁵. These patches comprise black earth intermixed with potsherds and stone stools; they are scattered throughout the Amazonian Basin and are classified as a soil type. Suggested explanations of the phenomenon include fallout from volcanos, sediments from vanished Tertiary lakes, remnants from slash-and-burn agricultural practices and cultural layers accumulated at former Indian villages. Smith argues persuasively that the first three explanations are not consistent with the observed geographical distribution or the depth and composition of the patches⁵. He concludes that the *terra preta* sites are the vestiges of ancient cultures in Amazonia, providing evidence that "precontact native populations were in many cases large and sedentary, particularly along rivers". If this is so, says Smith⁵, "biologists should be wary when they discuss virgin Amazonian ecosystems. Potsherds and black earth may lurk under control plots and pristine nature reserves".

Lifestyle

Not only may the early Amazonian populations have been more dense than previously thought, but it also seems likely that they were correspondingly more sophisticated. In particular, the fieldwork of Posey on the Kayapo Indians in Central Brazil has illuminated these peoples' rich appreciation of their environment⁶. The present population consists of about 2,500 Indians inhabiting 9 villages scattered within a 20,000 km² reserve.

Like most such people, the Kayapo have a large vocabulary to describe the subtleties of their surroundings. Their major subdivisions of the forest include *ba-kamrek* (riverine forest), *ba-epiti* (higher forest subject to intermittent flooding every 10 years or so, but containing some richer

alluvial soils), *ba-kati* (*terra firme*, lands that do not flood) and *ba-rarara* (forest with intermittent openings). Kayapo villages are sited to have access to a variety of these ecological zones, and the patterns of field ownership read like something from a current behavioural ecology text: although some fields are selected from *ba-kati*, others are carved from *ba-epti* because the better yields offset the unpredictable losses due to flooding; chiefs' fields and womens' collective fields are usually planted in such a way as to minimize total crop loss for any one family group. Not only do the Kayapo utilize some 250 plant species for their fruits, along with several hundred others for their nuts and tubers, but they also practice a transitional form of cultivation. In addition to cultivated plots near the villages, men on hunting treks rely on 'islands of resources' along their vast network of interlinked forest trails; these 'forest fields' are maintained by collecting plants during the day's travels and then replanting them near established campsites. At least 54 species of these semi-domesticated plants are used in the forest fields.

The Amazonian Indians also know their animals: "every significant detail of the life habits of animals is part of [the] hunter's knowledge, including the sound of its cry, its preferred foods, its excrement, its scents, the teeth marks it makes on fruit etc"⁷. Beyond this, the Kayapo practice a transitional form of domestication of animals. Thus, they deliberately stack the remains of banana and palm plants near villages to attract adult beetles that lay eggs in such refuse; in due course, the Indians know when to collect the tasty and nutritious grubs that mature from the eggs. The Kayapo further recognize some 54 species of stingless bees and 2 species of stinging bees: some of these are cultivated for honey and beeswax, and other species are cultivated (in rotting logs in holes dug in fields) as pollinators to increase crop yield.

Although little is known about the medicinal and other properties of the Kayapo's cultivars, at least two merit mention. The red seeds of *urucu* (*Bixa orellana*) are used to flavour foods and also as the main ingredient in body paint. Red body paint is widespread for adornment throughout Amazonia, but Posey also found *urucu* "to be an effective natural insect repellent with a significant reduction (as much as 84 per cent) in insect bites when painted on the body"⁶. The wild ginger *madn-tu* (*Zinziber*) is used — apparently with some effect — as a medicine against intestinal parasites.

Posey takes to task the common misconception that fields cultivated under slash-and-burn agriculture are totally abandoned after a few years. Kayapo 'abandoned' fields continue to produce harvests of yams and taro for 5–6 years, bananas for 12–15 and *urucu* for 20 years or more. The reforestation sequence also produces a variety of foods that attract

game in artificially high densities, and this phenomenon is also exploited. The Kayapo "thus do not have a clearcut demarcation between fields and forest, nor between wild and domesticated. Rather they have a more general system for classification of ecological resources that forms a continuum between wild and domestic ones, all of which figure into integrated management strategies"⁶.

Posey also tells some fascinating tales of the way myth and ritual interweave with natural history facts. One example must suffice. The myth: the little red ants are gentle like women and they are the relative/friend of the manioc; this is why women use the little red ant to mix with *urucu* to paint their faces in the maize festival; the little red ant is the guardian of the field. The facts: manioc has extrafloral nectaries that attract the ants, which in turn trim away any bean vines that would prevent the new and fragile manioc stems from growing; the beans can climb on the maize, which is sturdy enough to shoot up undamaged; and the leguminous beans help

fertilize the total crop. Thus, the coevolutionary relation of ants to the manioc-maize-bean system does indeed facilitate the women's work.

It is tempting to go beyond this, seeing such myth and ritual as functioning to regulate the Indians' use of natural resources. Although this may sometimes be so, I do not always find Posey's argument compelling: the Kayapo 'belief system' may simply serve to describe the world, rather than to manipulate it. Even so, such finely-nuanced description puts these people closer to the contributors to *Nature* than is commonly conceded. □

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Nomenclature

Code for Spanish names of birds

from I.F. Aguillo and M. Fernández-Cruz

ALTHOUGH publication of the International Code of Zoological Nomenclature was one of the most important achievements of modern taxonomy, vernacular nomenclatures remain useful as a communication tool; common names often hold a lot of information, as they represent a particular aspect of the relationship between man and his environment. The use of vernacular names is particularly common in the case of ornithology, a science with a high number of amateurs and much non-scientific literature. In that sense, ornithologists use both common and scientific names in their papers, giving special value to the former as an informative tool.

There is a great vernacular richness associated with birds, in every language and region of the world. The amount of information available is so large and varied, that quite often it is necessary to accept widely used names that are not as informative as they could be. This is not the case with the Spanish-speaking community of ornithologists, probably because the scientific infrastructure of the regions and the ornithological bibliography available in Spanish are very scarce. Thus the situation is favourable for the vernacular nomenclature of birds to be modified so that the names are more meaningful.

Since there are very few lists with accepted names in Iberoamerica, it will first be necessary to make a checklist to be used as a reference by all of the members of the community. Such a project has already been launched but its success will require the international cooperation of all 20 or so

countries integrated in this community. The ultimate aim is to produce a system of common names for the Iberoamerican and European avifaunas, based on associative and meaningful names.

General agreement has led to the proposal for a unified Code of Spanish common names which states the rules for deriving the new nomenclature. Although its criteria are inspired by the International Code of Zoological Nomenclature, informative names will be favoured over priority ones. Names will be brief and easy to write and pronounce; and will be selected on the basis of how informative they are.

The unified nomenclature will be binominal. The first word will be the 'generic name' which refers to all those species that can be grouped in accordance with taxonomical, morphological or historical-usage criteria. Preferably, the name should be derived from the vernacular roots of the community people, adapting it to the Spanish language. In other cases, the name will include information about ecology and ethology of the bird, but not morphological information. The 'specific name' will follow as an adjective, will characterize each species, and represent it in every way. This name will include information about the bird's morphology and appearance, geographical distribution and so on.

The Code and details of the proposed unification project, in Spanish, are available from the authors at: Cátedra de Vertebrados, Facultad de Biología, Ciudad Universitaria, 28040 Madrid, Spain. □

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A. Batters
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Solid-state perspectives of the photoelectrochemistry of semiconductor-electrolyte junctions

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More than one mechanism is required to account for the photoinduced movement of charge across semiconductor-electrolyte junctions. Distinguishing the various mechanisms, which is here accomplished using a synthesis of theoretical ideas from both solid-state physics and electrochemistry, may have important consequences in research related to potential applications of liquid junction devices in solar energy conversion.

PHOTOEXCITATION of semiconductors in contact with liquid electrolytes, followed by charge transfer of the photogenerated carriers into the liquid, is an important and well-studied phenomenon. This process can occur via several mechanisms which are generally not clearly differentiated in the literature. Furthermore, there are important similarities, as well as differences, between photoexcited semiconductor-electrolyte, semiconductor-vacuum, and semiconductor-solid systems. In this review, we discuss the mechanisms for photoexcited charge injection within a common framework, and connect the phenomena with other well understood semiconductor concepts. In addition, we provide a glossary of terms that joins the languages of solid-state physics and electrochemistry and will help readers of either discipline with the language of the other.

Two major phenomenological regimes can be identified. If the photoexcitation creates charge carriers with energies sufficiently large to be injected into continuum states of the liquid, then this process is defined as photoemission (PE). On the other hand, if the photoexcitation energy is sufficient to bridge the semiconductor band-gap, but insufficient for emission to occur into a continuum state of the liquid, the photogenerated carriers can still be injected into the liquid. This process occurs by charge separation and transport facilitated by the existence of a space charge (depletion) layer at the semiconductor-liquid interface; the process provides the basis for photoelectrochemistry. We call this process photoinduced charge transfer (PCT).

Photoemission

Photoelectric emission from a semiconductor into a vacuum is well understood. The simplest systems for analysis have no surface charge or space charge and their electronic states and transitions are illustrated in Fig. 1. The vertical axis represents total energy of an electron (eigenstate of the electronic hamiltonian) and the horizontal axis is position perpendicular to the surface. Within the semiconductor, the highest occupied band state at equilibrium is the top of the valance band E_v ; the lowest unoccupied state is the conduction band edge E_c ; the two are separated in energy by the forbidden gap E_g . The energy of an electron just outside the semiconductor, with zero kinetic energy, lies above E_v at the photoelectric threshold E_0 , often referred to as the vacuum level. The Fermi level E_f describes the thermodynamic occupancy of all electronic states, including dopant states within E_g .

In the photoemission measurements, the semiconductor is illuminated with photons of energy $h\nu$ and electrons are emitted into the vacuum and collected by a metal at an applied voltage

V with respect to the semiconductor emitter. For the metal there is no band-gap and E_f lies at the top of the occupied band states. For both semiconductor emitter and metal collector the work function W is the energy from E_f to the vacuum level. Two experimental conditions are shown: Fig. 1a for a voltage V_i between collector and emitter just sufficient for all emitted electrons to be collected; Fig. 1b, for a voltage V_e which only collects the most energetic electrons. From these measurements the photoelectric threshold E_0 can be obtained:

$$E_0 = W_e + E_f = h\nu + e(V_e - V_i) \quad (1)$$

where $h\nu$ is the photon energy. The work function of the emitter,

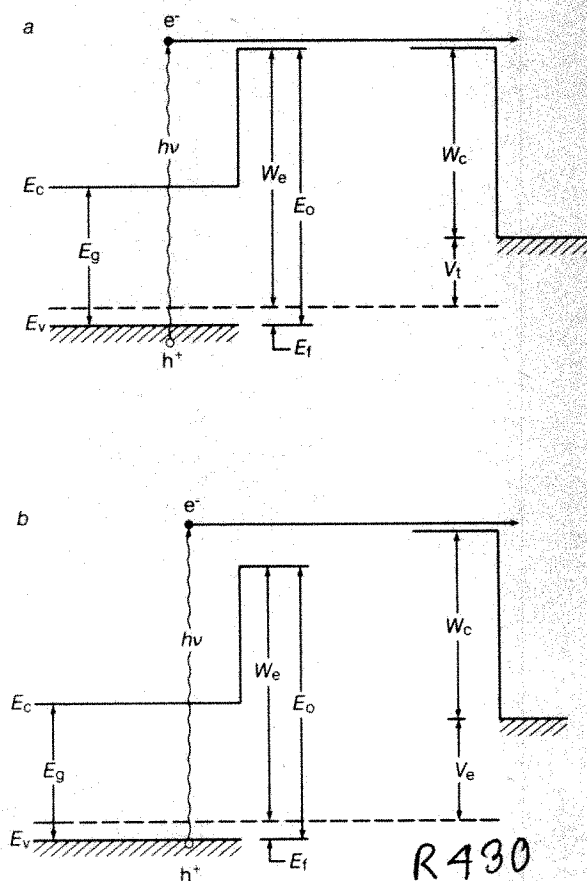


Fig. 1 Energy diagram for photoemission from semiconductors into vacuum: a, voltage that collects all emitted electrons; b, voltage that collects most energetic electrons.

* Deceased June 1984. Work performed while on sabbatical leave from Physics Department, University of Delaware, Newark, Delaware 19711.

The language of solid-state physics and electrochemistry

Acceptor dopant: An impurity which, in the neutral state, tends to accept an electron from the valence band, that is, to donate a positive hole.

Adiabatic potential: The effective potential for motion of atoms in a molecule or in a condensed phase, obtained in the approximation that the electrons move rapidly and the nuclei slowly. It consists of two terms: the nuclear-nuclear (or core-core) interactions, plus the eigenvalues of the electron state with parametric dependence on atomic coordinates.

Ballistic electron: An electron which traverses the active region of a device without generating or annihilating a lattice vibration (phonon).

Band structure: The relationship between the one-electron eigenvalues and the quasi-momentum which characterizes electronic states for crystals. For semiconductors a gap of forbidden eigenvalues lies between the occupied valence band and the empty conduction band. If the maximum of the valence band and the lowest minima of the conduction band are at the same value of the quasi-momentum, that is, Γ -points, the semiconductor is said to have a direct gap; if not, that is, lowest conduction minima at X- or L-points—an indirect gap.

Band tailing: The density of states for the band structure of undoped, perfectly-ordered semiconductors decreases rapidly to zero at the band edges. Dopants, disorders, space charges and applied electric fields perturb the density of states leading to tailing from the band edges into the forbidden gap.

Depletion region: The space charge region in a semiconductor which is depleted of majority carriers and produces a potential barrier for their transport to the interface.

Donor dopant: An impurity which in the neutral state tends to donate an electron to the conduction band.

Effective mass: Electronic particles in crystals react to an applied force as if they have an inertial mass which is different from the free-electron inertial mass. This mass is designated as the effective mass. The difference between the effective mass and the free-electron mass is due to the interaction between the electronic particle and the periodic potential of the crystal.

Electron-phonon interaction: The separation of the many-body problem of electrons plus nuclei in crystals into electronic band structure on the one hand and lattice dynamics (phonons) on the other is an approximation. A correction to this approximation involves coupling between the electrons in their states with the lattice dynamics and is called electron-phonon interaction. This interaction is essential for thermalization between electronic particles near their band

edges and for the molecular or lattice dynamics. It is also responsible for the polaron.

Exciton: An excited electronic state of crystals in which the excited electron and the positive hole are coupled (electron-hole pair); no net charge is transported during exciton motion. The effective mass exciton is a coupled conduction band electron and valence band hole: thus optical creation of effective mass excitons occurs on the long wavelength edge of the band-to-band optical absorption.

Fermi level: Energy level for which the occupational probability for an electron is 0.5, under thermal equilibrium conditions. The occupational probability for a positive hole is also 0.5 at equilibrium.

Franck-Condon principle: The most probable optical transitions between electronic states occur on a time scale short compared to the period of atomic motion, that is, molecular or lattice vibrations: the positions and momenta of the atoms remain fixed during these transitions. Franck-Condon transitions occur vertically between adiabatic potential curves for the initial and final electronic states of the transitions.

Helmholtz layer: The double layer of charge created at a solid-liquid electrolyte interface by the adsorption on the electrode of ionic and/or molecular species from the liquid.

Hot carriers: Electrons or holes that are not in thermal equilibrium with the lattice and have energies above the respective band edges, in the form of excess kinetic energy.

Negative electron affinity: Effect produced when sufficient band bending is generated in a p-type semiconductor with a low work function surface layer such that the position of the conduction band edge in the semiconductor bulk is above the vacuum level. Thus, photoemission of electrons into vacuum by band-gap photons can occur if this happens faster than intraband relaxation in the depletion region.

Polaron: For polar semiconductors, conduction band electrons and valence band holes do not move at their respective band edges as pure electronic particles but rather carry with them a cloud of lattice polarization (virtual phonons). The electronic particle bearing lattice polarization is itself particle-like and is called a polaron. The polaron mass is in general greater than the band effective mass, and quantitatively dependent on the electron-phonon interactions.

Positive hole: The ground state of a pure semiconductor has a filled valence band and an empty conduction band. States of excitation are thus best described focusing attention on the small concentration of electrons in the conduction band and/or the small concentration of unoccupied valence band states. The latter are electronic particles called positive holes, often abbreviated to 'holes'.

W_c is obtained from the first of these measurements if the work function of the collector, W_{c1} , is known¹.

The effects of space charge are exemplified by negative electron affinity photocathodes², shown for p-GaP in Fig. 2. Equilibration with several monolayers of n^+ -Cs₂O yields a large degree of band bending in the p-GaP, arising from the difference in work functions of these two materials and yielding a space charge. The eigenstates of the complete electronic hamiltonian, including the field due to space charge, are now more complicated than those described earlier, so that the position-dependent band edges E_c and E_v in the space charge region have a somewhat different interpretation than E_c and E_v in the space-charge-free region. In both regions, conduction electrons and positive holes (empty states in the valence band) which are in thermal equilibrium with the lattice move at energies near E_c and E_v , respectively. Band-to-band photoexcitation with photons bigger than E_g produces electrons with energies above the conduction band edge in the bulk. These electrons relax rapidly in <10 ps, in the flat-band region, and diffuse as thermalized carriers to the depletion region. From within this region, the photogenerated carriers can be emitted, without relaxing, to the conduction band edge E_c at the surface. Thus, electrons with energies in excess of the energies of thermal carriers in the depletion region, that is, non-equilibrated with the lattice at the GaP/Cs₂O interface, are emitted into the vacuum. Such electrons are one type of non-thermalized electrons, generally referred to as 'hot' electrons. Their ability to cross the depletion layer without thermalizing in that region, in contrast to the situation

in the flat-band region, results from the existence of a narrow depletion layer (<200 Å) and the associated large electric field.

Photoemission into a vacuum is irreversible because of the infinity of states in free space. Photoemission from a metal into a vacuum can be obtained from the above analysis by taking the limit $E_g \rightarrow 0$, where E_g is the band-gap.

Photoemission from a semiconductor into another phase of condensed matter is a valid concept if the electron emission is into continuum states, that is, into the conduction band, for example, of an aqueous electrolyte. The efficiency of emission is limited by differences in effective momentum k and mass m^* of the two phases. The concept can be generalized to include positive hole photoemission from the semiconductor into a continuum of hole states. These processes are illustrated in Fig. 3 for photons labelled $h\nu_{PE}$. The band edges and energy gap for the aqueous electrolyte are based on the model for liquid water as a large gap, lone-pair, amorphous semiconductor³. Figure 3 shows the effects of a space charge arising from the inequality of the work functions of semiconductor and electrolyte, $W_{sc} \neq W_{c1}$. For the p-type semiconductor, $W_{c1} < W_{sc}$; for the n-type semiconductor, $W_{c1} > W_{sc}$. These are the usual cases since $W_{sc} \approx W_{sc}^p + E_g$.

Electron or hole emission involves interchange between kinetic and potential energy; only total energy remains constant during the emission processes shown in Figs 1-3. The total energy for each electron divides as follows: the energy from the Fermi level, E_f , up to E_c is potential energy; from E_c to the eigenvalue E it is kinetic energy. Thermodynamically, the energy

Quantum well: An effective potential well created by a minimum in the conduction band or a maximum in the valence band that arises when a smaller band-gap semiconductor is sandwiched between a larger band-gap semiconductor. When the physical dimensions of the sandwich layers become small (comparable to the mean free path of electrons or holes), conduction electrons and positive valence band holes are localized in a dimension perpendicular to the layers because of quantization of the energy states in the well.

Quasi-Fermi level: The energy level for which the occupational probability for an electronic particle is one-half under steady-state conditions (time-independent but non-equilibrium). The quasi-Fermi levels for electrons and positive holes are different because of non-equilibrium concentrations of injected electronic particles, either electrons and/or holes.

Redox couple/redox potential: A redox couple is a pair of electron donor and acceptor species in solution that define a potential for charge transfer. At equal concentrations of donor and acceptor species (called the reduced and oxidized forms of the couple), the thermodynamic driving force for electron transfer to the acceptor or from the donor are equal. The couple is in equilibrium and this condition defines the standard redox potential, E_{redox}^0 , which is the electrochemical potential in the liquid electrolyte. The potential at other concentrations is related to E_{redox}^0 through the Nernst equation.

Solvated electron: A conduction electron in an electrolyte polarizes nearby molecules so that in this region the structure of the electrolyte is deformed and the electron acquires close ligands, that is, it is solvated. The solvated electron is, in fact, a polaron of rather large mass.

Space charge: The double layer of charge produced near the surface of a semiconductor in contact with a second phase resulting from the initial inequality of electrochemical potentials (that is, work function, redox potential, Fermi level) for the two phases. The charge is generally distributed over finite dimensions in the semiconductor.

Thermalized carriers: Electrons or positive holes that are in thermal equilibrium with the lattice, and move in semiconductors near the band edges E_c and E_v .

Work function: Work required to remove an electron from the Fermi level of a condensed phase into vacuum with the electron having zero kinetic energy. For liquid electrolytes the work function is identified with the redox potential of the electrolyte.

Zero-phonon transition: Optical transition between electronic states in which phonons are neither created nor annihilated. The transition normally occurs from the zero-point vibrational level of the initial state to that of the final state.

available to do useful work depends on an electron occupancy of state E in excess of thermal occupancies at the lattice temperature, and on $E - E_f$; the total available energy is independent of division into kinetic and potential energy. Similar considerations apply to the positive holes. With applied voltage and/or photoexcitation, E_f may for some conditions be described as split into two quasi-Fermi levels for electron and positive hole, E_f^e and E_f^h , respectively. The concept of quasi-Fermi levels is valid if occupancies of electron and hole states are separately in accordance with Fermi statistics.

Photoemission from the semiconductor into the forbidden gap, E_g^{el} , of the electrolyte modelled as an intrinsic semiconductor (undoped and free of defects) is obviated by the absence of allowed states within E_g^{el} . Here the electronic wavefunction ϕ attenuates exponentially in the electrolyte with a mean distance $\hbar/\sqrt{2m|E'|}$, where E' is measured from the band edge. Thus photoemission with electron energies within E_g^{el} can occur only within a range of a few molecular distances.

A more complete model of an aqueous electrolyte takes account of band tailing: that is, the allowed eigenstates tail into the forbidden gap. This shows itself in the exponential tail of the optical absorption of amorphous semiconductors and is evident in the vacuum ultraviolet absorption of water³. In general, band tailing can be explained in terms of the microfields generated by departures from a perfect periodic potential so that sharp E_c and E_v no longer exist⁴, or, alternatively, in terms of band edges which undulate spatially because of compositional and/or structural inhomogeneities⁵. Thus photoemission into

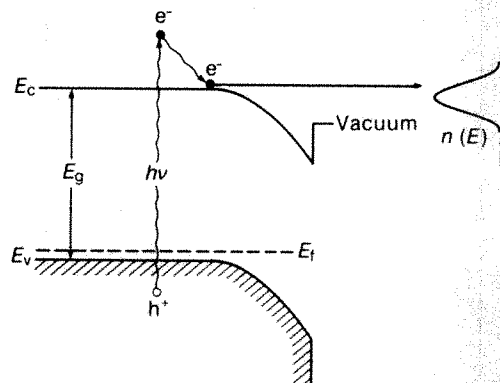


Fig. 2 Hot electron emission into vacuum resulting from band bending in negative electron affinity photocathode. Energy distribution of emitted electrons is shown (from ref. 2).

the band tails is predicted but with lower probability because of the low densities of states.

Another mechanism specific to liquid electrolytes is photoemission directly into solvated electron states⁶. This depends on the molecular polarization and orientation in the vicinity of the trapped electron. The lowest of these states in water lies about 2 eV below the unsolvated electron states. The solvated electron is a low mobility polaron, a quasi-particle well known in the solid state. Because electron emission is fast compared to intermolecular motion, photoemission directly into solvated electron states depends on the electrolyte preparing itself spontaneously in a state of polarization consistent with the solvated state. Since liquid electrolytes are at equilibrium, the probability of such pre-transfer polarization into states 2 eV below the band edge is quite unlikely. Similar considerations apply to the possibilities for photoemission into self-trapped positive hole states of the electrolyte.

Photoinduced charge transfer

Photoinduced charge transfer (PCT) is the process wherein electron-hole pairs are first created in the conduction and valence bands of semiconductor electrodes by absorption of photons with band-gap energies; then separated by the field in a depletion layer at the semiconductor-electrolyte interface; finally electrons or holes are injected into the electrolyte to drive redox reactions. For n-type semiconductors, the holes move to the semiconductor-electrolyte interface to generate an oxidation reaction (semiconductor is a photoanode); for p-type semiconductors, electrons move there to generate a reduction reaction (semiconductor is a photocathode). Photoinduced electron transfer is illustrated in Fig. 3a and hole transfer in Fig. 3b for photons labeled $h\nu_{\text{PCT}}$: PCT can occur from carriers with energies near the band edges in contrast to the energy required for photoemission. In addition, under certain conditions, fast PCT by direct injection into dopant or defect states of the electrolyte may be possible. Processes involving PCT have been extensively studied in recent years, and many reviews are available⁷⁻¹⁰.

The impetus for this research is the potential application of liquid junction devices to solar energy conversion⁷⁻¹⁰. Photoelectrochemical cells containing photoactive semiconductor electrodes can be used either to drive redox reactions (for example, water splitting, CO_2 reduction, and N_2 reduction) producing fuels and chemicals, or in electrochemical photovoltaic cells to produce electricity.

The important distinction between PCT and photoemission is that in the latter the photogenerated electrons or holes are injected into the continuum states of the electrolyte, whereas in PCT the photogenerated carriers are injected into dopant or defect states that exist in the band-gap of the electrolyte. Dopant states are associated with the redox potential of redox couples added to the liquid, whereas defect states are redox potentials associated with the liquid itself. The latter are the native defects of the liquid electrolyte; for example, H^+ and OH^- ions in liquid

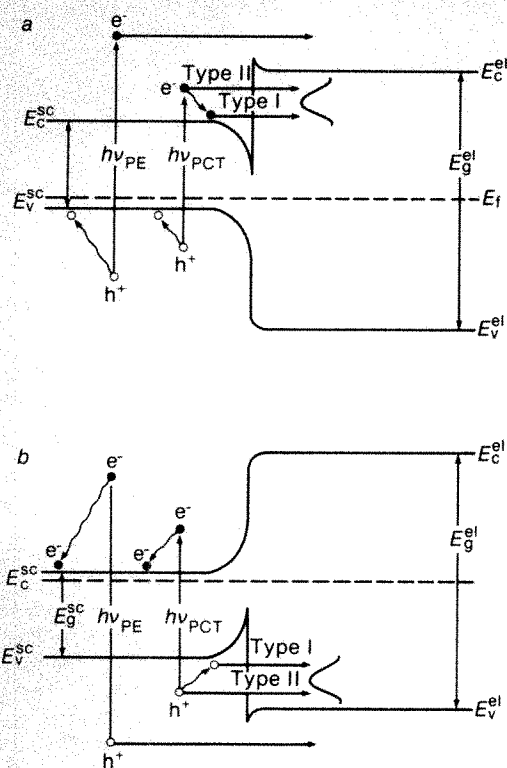


Fig. 3 Photoemission and photoinduced charge transfer (PCT) processes from semiconductors into liquid electrolytes: *a*, for electrons; *b*, for holes. Photoemission occurs with $h\nu_{PE}$; PCT occurs with $h\nu_{PCT}$. The PCT processes are shown for Type I and Type II hot carrier injection. Thermalized PCT processes inject the carriers from the bottom of the semiconductor conduction (or valence) band at the semiconductor-liquid interface. The energy distribution of the electron acceptor in the electrolyte is shown, and arises from fluctuations of the solvation shell around the acceptor (see Fig. 4 for further details).

water. Thus, the distinction is based primarily on the nature of the relevant electronic states of the electrolyte, rather than on intrinsic differences in the nature of the photoprocesses in the semiconductor. This is evident in Fig. 3.

In PCT, the carriers injected into the electrolyte can either be fully thermalized in the semiconductor or not. When thermalized, the photogenerated electrons and holes arrive at the interface with energies corresponding to the band edges E_c and E_v at the surface, respectively. These carriers always remain in equilibrium with the lattice through electron-phonon interactions, and dissipate the kinetic energy created by band-bending in the depletion layer as heat, as the carriers traverse the depletion layer. In thermalized PCT, only redox potentials that lie within E_g of the semiconductor at the interface are accessible to the injected charge.

For the case of non-thermalized PCT, illustrated in Fig. 3, the photogenerated carriers are injected into the electrolyte before they have undergone complete thermalization as they move from bulk semiconductor into the electrolyte. This process, called hot carrier injection, has been analysed in some detail¹¹⁻¹⁴, and is discussed further below. For hot carrier injection, redox couples that have potentials outside E_g of the semiconductor at the liquid contact are accessible.

Finally, we discuss the role of surface states. In semiconductor-vacuum and semiconductor-solid interfaces, the major effect of surface states is to pin the Fermi level so that the band-bending is independent of, or insensitive to, the nature of the second phase contacting the semiconductor. In semiconductor-liquid interfaces, the nature and effects of surface states can be quantitatively and qualitatively different from those of solid state junctions. First, ionic species in the electrolyte can be specifically

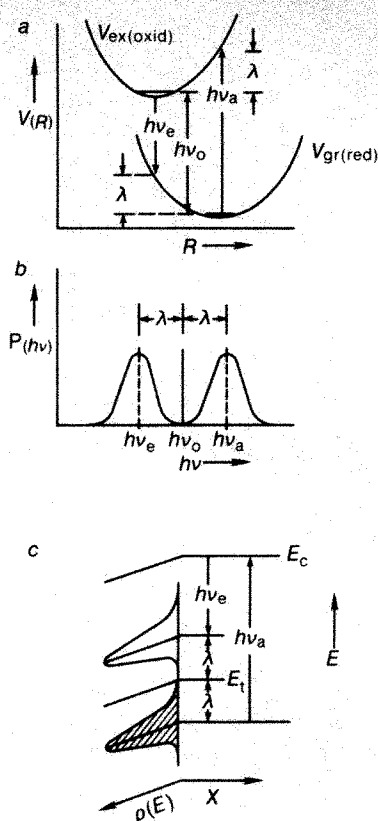


Fig. 4 *a*, Adiabatic potentials for ground (reduced) and ionized (oxidized) states of dopants in polar semiconductors, insulators or electrolytes, with Franck-Condon and zero-phonon transitions. *b*, Probability distributions for radiative transitions. *c*, Electronic energy levels, with thermodynamic dopant level E_t and effective densities for transitions. For redox couples in electrolytes E_t is identified with E_{redox}^0 (see box).

adsorbed onto the semiconductor either to passivate surface states or create new ones¹⁵. Second, Fermi level pinning or surface state charging under illumination can move the relative energy levels of the semiconductor with respect to those of the liquid electrolyte, and hence affect the kinetics of charge transfer¹⁶⁻¹⁸. Finally, surface states can mediate the charge transfer such that it occurs from the surface state rather than from the semiconductor band edges²⁰; this could also have a very large effect on the kinetics of PCT.

Large-gap amorphous semiconductors

Liquid electrolytes such as water can be described, with respect to electronic states, as large-gap amorphous semiconductors. The optical properties of pure and doped liquid water have been explained with this model³. It also provides the basis for interrelating some electrochemical concepts (for example, redox couples) with semiconductor concepts (for example, donor and acceptor dopants). However, semiconductor theoretical analyses of electrolyte-semiconductor junctions require modification to take account of the large density of mobile ions in liquid electrolytes.

Charged point defects in crystalline semiconductors or insulators are well known to have bound states for electronic particles: bound electron states for a positively-charged defect and bound hole states for a negatively-charged defect—termed donor and acceptor dopants²¹ respectively. Some uncharged dopants bind an electron or a positive hole and are referred to as isoelectronic dopants²².

These dopants in polar semiconductors or insulators have bound states whose energies are dependent on occupancy. This is a consequence of electron-phonon interaction and can be

visualized in detail by considering the adiabatic potentials $V(R)$ for two states of occupancy, as shown in Fig. 4a. $V(R)$ is the interaction potential between the atoms, in the region of the dopant with position coordinates $R_1, R_2, R_3, \dots$, symbolically represented by the single coordinate R , and is dependent on electronic state. Thus, the equilibrium value for R is different depending on whether or not an electronic particle is bound to the dopant. Optical attachment or detachment of the particle in accordance with the Franck-Condon principle (see standard texts, for example, ref. 23, and box) will be followed by lattice relaxation from the equilibrium position of the initial state to that of the final state.

The $V(R)$ are assumed harmonic with the same force constant for the ground and excited electronic states; the change in polarization energy is designated λ (anticipating identification with change in solvation energy for ions in electrolytes); $h\nu_0$ is the zero-phonon transition energy and equals E_i , which determines the thermodynamic occupancy of the two states; $h\nu_a$ and $h\nu_e$ are the Franck-Condon transitions for optical absorption (ionization) and emission (deionization), respectively. In Fig. 4b the probability distribution for these transitions is shown. Their band widths and Stokes shift are consequences of zero-point and thermal occupancy of the initial state, and of the transformation from the R -coordinate to transition energy.

These states and transitions can be approximately represented on an electronic energy level diagram as shown in Fig. 4a. We emphasize, as have others²⁴, the differences between $V(R)$ and electronic energy E . E_i lies at that energy below the conduction band edge E_c , with the approximation that E_c is independent of local polarization. For Franck-Condon transitions, the effective densities of states, $\rho(E)$, for ionization and de-ionization are respectively displaced, deeper and shallower by λ , and also broadened. The $\sqrt{E - E_c}$ dependence of $\rho(E)$ for the conduction band is also shown.

The same phenomena occur with redox couples in liquid electrolytes. The two states of occupancy correspond to reduced and oxidized forms of the couple, that is, dopant, similar to those shown in Fig. 4. The structure of the liquid does not have translational periodicity and therefore its lattice dynamics are not describable by phonons; however, the relaxation process is describable in terms of local modes and is basically the same as if the matrix for the dopant were crystalline. The $V(R)$ for each electronic state of occupancy or of excitation is characterized by different equilibrium molecular coordinates R , now in the solvation shell; λ is the energy associated with the reorganization of the solvent molecules around the redox species as it becomes oxidized or reduced. In general, the force constants are different but here are approximated as the same. The energy distributions of oxidized and reduced forms of a redox couple can be related respectively to those of ionized donor (or neutral acceptor) and those of neutral donor (or ionized acceptor) of point charged semiconductor dopants. However, the non-radiative charge transfer transitions for redox couples in electrolytes have different energy dependences than radiative charge transfer transitions for dopants in semiconductors if the former involve prior reorganization of the coordination shell: that is, if the transfer occurs adiabatically rather than vertically. This is, of course, basic to the Marcus theory²⁵. On the other hand, Franck-Condon effects have been effectively invoked by Gerischer²⁶ to describe charge transfer at metal-electrolyte and semiconductor-electrolyte junctions. Tunnelling of electrons or positive holes from electrolyte to semiconductor or metal in accordance with the Franck-Condon principle, that is with fixed atomic coordinates, will be at the energies and with the band widths of Fig. 4.

The transfer of electronic charge between a redox couple and an electrode involves only the states of the couple, not those of either free or solvated electrons, and thus the thermodynamic potential of the couple, E_i of Fig. 4c, will be at the Fermi level of the electrode. Ionic transport maintains continuity of current to the redox couple. This differs from charge transfer in semiconductors, for which Fermi levels are determined by conduction

and valence band edges, as well as by dopant states; these band states are necessary for current continuity because of the absence of significant ionic transport in most semiconductors. The explanation of this difference is that the dopants in liquid electrolytes are fully compensated by other dopants or by native defects. Thus conduction electrons or valence band holes are not present in normal electrochemical processes; they are unnecessary in view of the presence of mobile ions. In other words, for PCT the redox couple is decoupled from the band structure of the electrolyte, whereas the semiconductor dopant is not decoupled from the band structure of the semiconductor. However, for photoemission the relationship of the states of the redox couple to the band structure of the electrolyte is, of course, important; in this case the redox couple is not decoupled from the electrolyte band structure.

The difference between semiconductor-electrolyte junctions and semiconductor-semiconductor junctions is thus evident for PCT. For semiconductor-electrolyte junctions, electrons or positive holes can be injected into redox couples and continuity of current satisfied by ionic transport; for semiconductor-semiconductor junctions electron or positive hole injection from conduction or valence band states of one semiconductor into dopant states within the gap of the other semiconductor is obviated by the absence of continuity of current owing to the immobility of the dopants and the absence of inter-dopant charge transport. However, for photoemission the relationship of the states of the redox couple to the band structure of the electrolyte is, of course, important.

Finally, semiconductor-liquid electrolyte junctions involve space charge, in the electrolyte from accumulation or depletion of mobile ions, and in the semiconductor from electronic charge transport for equilibration¹⁴. The electrolyte is charge compensated so that its Fermi level lies near the middle of its energy gap. For moderate electrolyte concentrations, the mobile ions lead to a narrow space charge, that is, Helmholtz layer, similar, as regards high density of space charge, to a junction involving a semiconductor doped to degeneracy. Dilute electrolytes lead to a wider space charge analogous to low doped semiconductors.

Hot carrier photoinduced charge transfer

Mobile electronic particles are normally equilibrated with the lattice modes; thus, occupancy of the electronic states is characterized by the same temperature as that for occupancy of phonon levels. Exceptions occur under severe perturbations, for example, high electric fields, during which energy is acquired by the electronic particles faster than thermalization with the lattice occurs. In this way the energies of the electrons and/or holes increase and the particles are referred to as hot although their energy distribution may not in general be describable by a temperature. If the density of the carriers is high, that is, $\geq 10^{14} \text{ cm}^{-3}$, collisions among carriers are sufficiently frequent for the distribution to become maxwellian²⁷; otherwise the carrier distribution is non-maxwellian. A well-known example is the displaced maxwellian for an electron gas in an applied electric field, in which the normal velocity distribution is shifted for its velocity component in the direction of the field. Photogenerated hot carriers have, of course, initial distributions dependent on the spectrum of the photoexcitation: for example, with a monochromatic source and excitation limited to transitions with $\Delta k = 0$, the initial photo-electron and photo-hole distributions will each be a δ -function. For dilute electrons in intense electric fields, over distances less than the mean free path for phonon scattering, ballistic motion occurs. This phenomenon has been reported for short semiconductor devices²⁸.

The energy distributions of photogenerated conduction electrons and valence band holes, in the region of a semiconductor-electrolyte junction, depend on the photoexcitation spectrum, on intraband relaxation, on interband electron-hole recombination, on the rate of charge transfer from semiconductor to electrolyte, and on the dependence of that rate on electronic energy. The magnitudes of these rates have been estimated, with attention to the effects of quantization of electronic states in

narrow depletion layers on intraband relaxation^{11,13}, and hot carrier transfer predicted from states below the band edge of the bulk semiconductor but higher than thermal energy above the edge at the interface. These are defined as Type I hot carriers. Type II hot carriers are distinguished as having energies more than thermal energy above the band edge of the bulk semiconductor. These processes are illustrated in Fig. 3.

Transfer of photoexcited carriers across semiconductor-electrolyte interfaces have been analysed using the master equation¹³. The total current depends on charge transfer versus recombination. The energy distribution of the charge transferred depends, in addition, on relaxation, density of depletion layer states, and the energy 'window' through which transfer occurs to the redox couple. Back transfer is assumed negligible in this analysis.

Experimental evidence for Type I hot electron injection across illuminated semiconductor-electrolyte interfaces has been obtained for p-GaP (ref. 29) and p-InP (ref. 30) electrodes in non-aqueous electrolyte. In these experiments, reduction reactions of electron acceptor species having redox potentials above E_c at the surface are observed. Such reactions are called supra-band-edge redox reactions, and can only occur via a hot carrier process if the semiconductor band edges do not move with respect to the electrolyte redox levels, during the experiment. The absence of band-edge-movement was established through monitoring of the band-edge energies by capacitance measurements. For p-GaP, the electron acceptor was anthracene (0.9 eV above E_c), and for p-InP, the electron acceptor was p-nitrobenzonitrile (0.5 eV above E_c).

Quantization in small particles

Size quantization effects, previously discussed for narrow depletion layers in planar semiconductor-electrolyte junctions^{11,13}, and well established for semiconductor quantum wells^{31,32} are also evident for small semiconductor particles (20–200 Å diameter) suspended in electrolytes^{33–38}. For the latter, the conduction and valence bands are separately fully quantized in three dimensions into discrete states, and the effective band gap increased as the particle size becomes very small. This essentially produces a hot carrier effect in that the kinetic energy is increased by the confinement. The effect is dependent on effective mass, and is thus material dependent; size quantization effects appear with larger particle sizes for semiconductors with smaller electron effective mass. For the same material, the confinement effect is different for different extrema of the band structure³⁸; for example, the low effective mass Γ -conduction band minimum of tetrahedral semiconductors is perturbed more than the higher effective mass X- and L-band minima. For quite small particles of direct-gap tetrahedral semiconductors, size quantization perturbs the effective band edges into the non-parabolic region of the Γ minimum. For very small particles, for example, ~20 Å for CdS, degeneracy of the three conduction band minima is predicted³⁸.

In addition to the separate perturbations of the conduction and valence bands, small dimensions may result in correlation between the electron and positive hole when they are confined to the single particle. One consequence is that the internal energy of excitons created during photoexcitation increases as the confinement dimensions (D) become comparable to or less than the Bohr radius of bulk excitons (r_B), for example $D \leq r_B \approx 100$ Å for GaAs (refs 36–38). This effect is well established for laminar semiconductor quantum wells³⁹. For small semiconductor particles suspended in electrolytes, the effect of correlation on the band structure itself also becomes appreciable as a result of the electron and hole being confined to a single particle. The effects of correlation on band structure and excitons both depend on occupancy of states, and are present or not depending on the details of the optical experiment; for photoexcitation, the accessible electronic states, whether originating from band or excitonic states, are affected by the Coulomb correlation of electron and hole; for photoemission or fast PCT, the accessible

states are, to a good approximation, band states quantized by confinement, but quite distinct from excitonic states.

In general, for semiconductors with low effective mass electrons, the confinement perturbation is greater than the Coulomb correlation. Thus, for photoexcitation, photoemission or PCT the general features of bulk band structure are predicted to survive in quite small particles, with the forbidden gap increased.

Some of these and related effects have already been found experimentally, for example, the predicted blue shift in absorption spectra^{33,36,37}. However, the distribution of particle sizes in currently available experimental samples gives rise to broad spectra, rather than the discrete spectra predicted theoretically for particles of uniform size and shape.

Size quantization effects with small semiconductor particles in electrolytes should lead to important modifications in the nature of charge transfer and the attainable power conversion efficiencies in photochemical devices: for example, shifts in effective photochemical redox potential have been predicted^{33–38}. Also, unusual kinetics are expected with photoexcitation of very small particles: for example, electron-hole recombination will occur in pairs (geminate recombination), with each pair confined to a particular particle, rather than by the second-order process of bulk semiconductors³⁸.

Conclusions

Photoelectron emission and photoinduced charge transfer at semiconductor-electrolyte junctions can be clearly distinguished. These junctions can be described by semiconductor heterojunction theory if modifications are made to include the effects of charge compensation and of the mobile ions in the electrolyte. Dopants in polar semiconductors and redox couples in electrolytes are similar as regards polarization changes on occupancy but differ as regards equilibration with band edges. Surface states can play an important role in semiconductor-electrolyte junctions in that in addition to the possibility of pinning the Fermi level as in solid state junctions, they may interact strongly with the electrolyte, cause band movement with respect to the electrolyte redox potential, and mediate charge transfer to the electrolyte. Hot electrons were predicted theoretically and found experimentally. Two types of hot carriers can be distinguished on the basis of their electronic kinetic energies in depletion layers and in the semiconductor bulk, respectively. Quantization effects can occur in narrow depletion layers and in small semiconductor particles. This can cause major changes in optical properties and charge transport.

This work was funded by the US Department of Energy, Office of Basic Energy Sciences, Division of Chemical Sciences, under Contract EG-77-C-01-4042. We acknowledge helpful comments by B. A. Parkinson and J. A. Turner. This paper is dedicated to the memory of Ferd Williams.

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ARTICLES

Detection of a radio emission at 3 kHz in the outer heliosphere

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A radio source in the outer heliosphere has been detected by the plasma wave receivers on Voyagers 1 and 2. The radio emission is observed in the frequency range 2-3 kHz, and is above the local solar wind electron plasma frequency whenever supporting plasma density data are available. The maximum spectral density of the emission recorded is $\sim 10^{-14} \text{ V}^2 \text{ m}^{-2} \text{ Hz}^{-1}$. The bandwidth of the radio noise is $\sim 1 \text{ kHz}$. Possible sources include continuum radiation from Jupiter's distant magnetotail and radiation at the second harmonic of the plasma frequency at the heliopause. If the latter interpretation is correct, these data represent the first remote observations of the heliopause.

THE Voyager 1 and 2 spacecraft are currently sampling the interplanetary environment at heliocentric radial distances of 19 and 13 AU, respectively. Since the Saturn encounters in 1980 and 1981, the plasma wave data have shown very little activity in the outer heliosphere. The primary waves observed include occasional electron plasma oscillations and ion-acoustic waves, sometimes detected in association with interplanetary shocks. Figure 1b shows the trajectories of Jupiter, Saturn, Uranus and Voyagers 1 and 2 for the interval 30 August 1983 to 21 February 1984; during this time a radio emission near 3 kHz was being detected by the plasma wave instruments on both Voyagers. Figure 1a shows a meridional view of the outer planets and the trajectories of the two Voyagers. In this view, the abscissa is in the ecliptic plane and the positions of the various planets and spacecraft have all been rotated into the same plane.

The observations discussed here were obtained on both Voyager spacecraft using identical plasma wave receivers which measure the electric field component of plasma and radio waves in the frequency range 10 Hz to 56 kHz by the use of 16-channel spectrum analysers and wideband receivers with passbands of 40 Hz to 12 kHz (ref. 1).

Observations

During the period from 30 August 1983 to the present, a very weak signal has been apparent in the 3.11-kHz channel of the Voyager 1 plasma wave instrument. The 3.11-kHz data for a 6-month interval are plotted as a function of time in Fig. 2. Each point in Fig. 2 represents a 51.2-min average of the electric field spectral density in the 3.11-kHz channel. It is apparent that after about day 242 (30 August) 1983 a smoothly varying signal was continuously present. Amplitude variations within the 51.2-min averaging intervals were small compared with the long-term variations seen in Fig. 2. Beginning at about day 292, the variations appear to be quasi-periodic with a period of about 26 days. This is close to the sidereal period of the Sun, hence, we suspect the solar wind may be responsible, in part, for the amplitude variations. There also seems to be a gradual overall variation in the amplitude of the emission with a rise from the onset near day 242 to the peak near day 285 and a subsequent

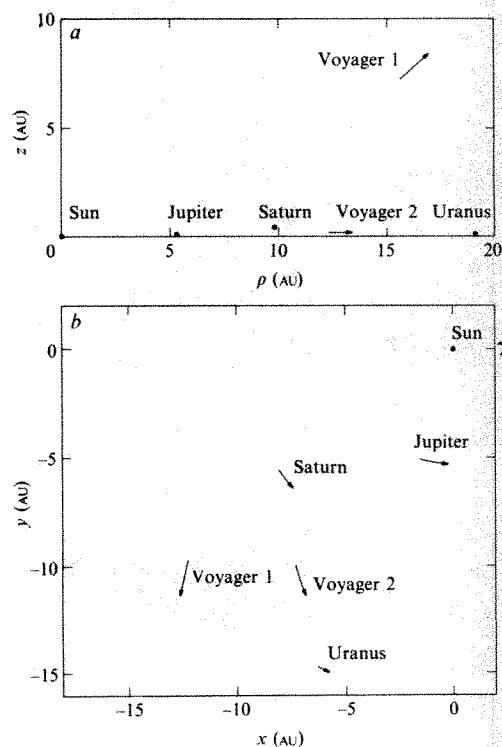


Fig. 1 The trajectories of Jupiter, Saturn, Uranus, and Voyagers 1 and 2 during the interval 30 August 1983 to 21 February 1984 when the Voyager 1 plasma wave receiver was detecting a new radio emission in the frequency range 2-3 kHz. Evidence from Voyager 2 indicates the radio emission may have been observable from that vantage point over a similar interval. *a*, The positions of the outer planets and the Voyager spacecraft rotated into a single meridian plane with the ρ -axis lying in the ecliptic plane. *b*, The projection of the trajectories of the same bodies in the ecliptic plane with the x -axis pointing in the direction of the first point in Aries, γ .

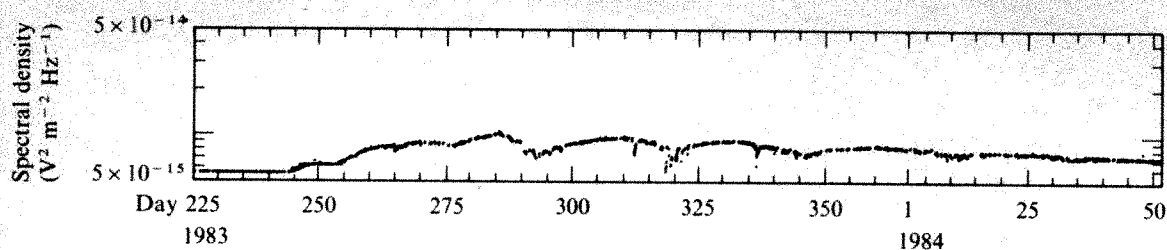


Fig. 2 Electric field spectral densities from the Voyager 1 3.11-kHz plasma wave receiver channel plotted as a function of time for slightly more than six months. Each point represents a 51.2-min average. The radio emission first appeared on day 243 1983.

decrease in amplitude towards the end of the plotted interval. During this entire time interval, no signal was detected in the 5.62-kHz channel. For a few one- or two-week periods a much weaker emission could be seen in the 1.78-kHz channel.

Several times during the interval plotted in Fig. 2, the wideband channel of the Voyager 1 plasma wave receiver was interrogated. For each wideband interrogation, four contiguous 48-s 'frames' of plasma wave data with high temporal and spectral resolution were obtained. In each wideband frame transmitted after day 242 1983, a weak band of emission near 3 kHz could be seen. Table 1 summarizes the wideband observations of the 3-kHz emission by both Voyager spacecraft. SCET is the spacecraft event time for each wideband observation and f_0 and $f_{\min}$ are the frequencies of the peak of the emission and the low-frequency cutoff, respectively. The electron plasma frequency as determined by the Plasma Science instrument (J. W. Belcher, personal communication) is labelled f_p .

An example of one of the wideband frames is shown in Fig. 3 in the format of a frequency-time spectrogram where the amplitudes of waves are plotted as a function of frequency and time. The most intense waves are black. In Fig. 3, the intense tones at frequencies below 1 kHz are interference from the operation of the onboard tape recorder used to record the wideband data. The very narrow tones at 2.4 and 4.8 kHz are the first and second harmonics of the spacecraft power supply.

The diffuse noise seen between 2 and 3 kHz is the emission which accounts for the signal received in the 3.11-kHz channel plotted in Fig. 2. This spectrogram is typical of the others received since day 242 1983, except for variations in the signal strength and minor variations in the lower frequency cutoff of the emission. The data from a 4-s interval in the spectrogram in Fig. 3 were averaged to form the spectrum shown in Fig. 4a which illustrates the detailed structure of the emission band. The power supply interference tone at 2.4 kHz, which rises out of the middle of the emission band, and a notch filter which serves to limit the power supply interference, cause some difficulty in the interpretation of the spectrum in Fig. 4. The gap

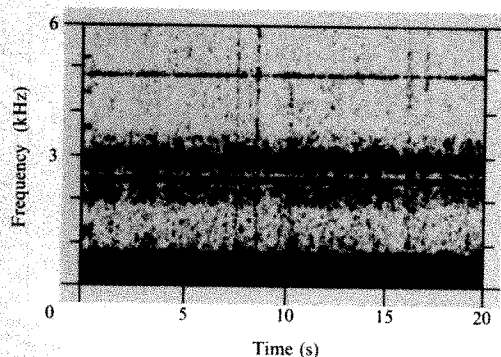


Fig. 3 Spectrogram showing the intensity of waves as a function of frequency and time for a 20-s interval. The diffuse emission seen between 2 and 3.5 kHz is the new radio emission reported here. The narrowband tones at 2.4 and 4.8 kHz as well as below about 800 Hz are spacecraft interference. Start time 2021: 52 SCET of day 307, 1983; $R = 17.9$ AU; celestial lat. = 25.5° .

in the spectrum at 2.4 kHz may be due to the notch filter, but we cannot rule out the possibility of a complex spectral structure which is inherent in the radio emission itself.

The Voyager 2 3.11-kHz channel sensitivity is not as great as that on Voyager 1 because of a partial failure in the Voyager 2 Flight Data System which occurred shortly after launch. Hence, at these low wave amplitudes we do not see any response in the Voyager 2 spectrum analyser channels. On the other hand, wideband frames obtained from Voyager 2 (which are unaffected by the Flight Data System failure) show a feature which is very similar to that shown in Fig. 3 (see Table 1). The earliest Voyager 2 wideband frame showing evidence of the emission was obtained on day 257 (14 September) 1983 when the spacecraft was 12.7 AU from the Sun. Samples showing evidence of the emission were also obtained on days 294, 307, and 339 of 1983 and day 3 of 1984. Because no wideband frames were obtained on Voyager 2 between day 228 (when the emission was not seen in the wideband data) and day 257, it is possible that the signal has been continuously present at Voyager 2 since about day 243 as was the case for Voyager 1. This close correspondence in onset times observed at two locations separated by nearly 10 AU suggests the two spacecraft are detecting a temporal change as opposed to a spatial variation.

Figure 4b shows a spectrum taken on day 307 by the Voyager 2 plasma wave instrument. The receiver threshold is not quite as low as in the case of Voyager 1, but the similarity between the emission just below 3 kHz with the feature shown in Fig. 4a can be readily seen. Note that the amplitude of the emission is nearly identical to the emission observed by Voyager 1.

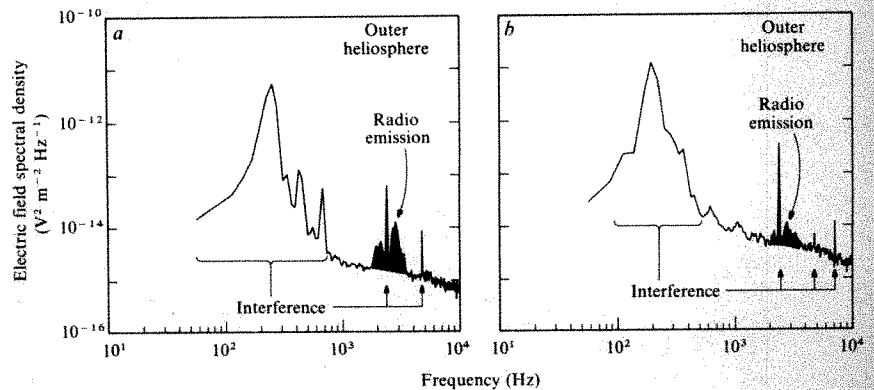
The Voyager 2 observations of the emission are important in our identification of the emission as a radio signal because the plasma instrument² on Voyager 2 can provide a measure of the local electron density, hence, plasma frequency. (The Voyager 1 plasma instrument has not functioned since shortly after the Saturn encounter.) If the emission is a freely propagating radio emission, then it must lie above both the electron plasma frequency and gyrofrequency. For four of the five days when wideband data are available, a determination of the density from the plasma instrument yielded values ranging from 0.02 to 0.04 cm^{-3} as shown in Table 1 (J. W. Belcher, personal communication). The respective plasma frequencies range from

Table 1 Wideband waveform observations of 3-kHz radio emissions

Year	Day	SCET	f_0 (kHz)	$f_{\min}$ (kHz)	f_p (kHz)
Voyager 1					
83	283	1407	2.8	1.9	NA
83	307	2022	2.9	1.8	NA
83	339	1720	3.0	1.9	NA
84	3	1619	3.0	2.1	NA
Voyager 2					
83	257	1940	3.0	2.7	1.4
83	284	1452	2.8	1.9	NA
83	307	1633	2.8	2.0	1.3
83	339	1747	3.0	2.6	1.3
84	3	1805	3.1	2.7	1.8

NA, not available.

Fig. 4 *a*, 4-s average spectrum taken from the spectrogram shown in Fig. 3 at 2021: 56 SCET. The shaded portion of the spectrum corresponds to the new emission. Note that the interpretation of the spectrum is complicated by the existence of a notch filter and power supply tone centred at 2.4 kHz. *b*, A 4-s average spectrum of the 3-kHz radio emission observed by Voyager 2 at a time (1632: 05 SCET) very close to when the emission shown in *a* was observed by Voyager 1. Note the similar amplitude of the features near 3 kHz in *a* and *b*.



1.3 kHz to ~ 1.8 kHz because $f_p(\text{Hz}) = 8,980\sqrt{n_e}$, where n_e is the electron density in cm^{-3} . For days 257, 307, and 339, the values for f_p are obtained from the 3-min determination of n_e made closest to the wideband frames and have errors of $\sim 10\%$. No reliable measurement is available for day 284 and the value for day 3 of 1984 is a daily average used in the absence of detailed analyses which are not yet completed. The low-frequency cutoff of the emission ranged from ~ 2 to nearly 3 kHz over the five intervals of wideband data. In each case, the emission exhibited a low-frequency cutoff which was well above the local electron plasma frequency. As the emission lies above f_p and the electron gyrofrequency in the solar wind is much less than f_p , the emission meets a necessary condition for being a freely propagating radio wave. It is easy to speculate that the periodic dips in the emission amplitude seen in the Voyager 1 data plotted in Fig. 2 are due to the passage of relatively high-density structures in the solar wind associated with high-speed streams which shield the spacecraft from the source of emission.

With the available data, it is not possible to confirm that the emission is a freely propagating radio wave; however, we can eliminate local plasma phenomena with a high level of confidence. There are two types of local (non-propagating) phenomena which have been observed at, or above, the local plasma frequency in the solar wind. The most familiar waves in this frequency regime are electron plasma oscillations or Langmuir waves. The observed waves are almost certainly not plasma oscillations as the bandwidth of the observed emission is much larger than that for plasma oscillations, particularly at low amplitudes and plasma oscillations are characterized by a very sporadic temporal character. The observed emission shows only very smooth variations in amplitude with time.

The second type of local phenomena which might be considered are quasi-thermal electrostatic plasma waves⁷. Hoang *et al.*³ discuss the spectral form of the thermal plasma emission in detail for both long and short (compared with the Debye length) antennas. For the typical solar wind observed in this epoch by the two Voyagers, the density is 0.05 cm^{-3} , hence, even with a temperature as low as 1 eV, the Debye length is > 30 m, compared with an effective antenna length on the two Voyagers of 7 m. Therefore, the short antenna results of Hoang *et al.* are relevant to this discussion. The quasi-thermal plasma noise detected by a short antenna has a power law spectrum with an index of -1.5 and shows no peak and no cutoff at f_p . The spectra shown in Figs 3 and 4 exhibit both a low-frequency cutoff and at least one peak. These waves, therefore, are clearly not the thermal electrostatic waves discussed by Hoang *et al.*³.

Table 1 also shows that the frequency of the peak in the emission is nearly the same at both spacecraft despite the relatively large variations in local plasma conditions which would be expected between two points separated by ~ 10 AU. The nearly constant frequency would not be expected if the emission were a local effect. Finally, the fact that Voyagers 1 and 2 both began detecting the 3-kHz emission at about the same time despite being nearly 10 AU apart argues strongly for a freely

propagating radio emission and against a local plasma phenomenon.

Possible sources

Several possible sources exist in, and beyond, the outer heliosphere for the radio emission reported here. The emission is most reminiscent of continuum radiation trapped in Jupiter's distant magnetotail because it lies in a very similar frequency range. Figure 5 shows an example of the continuum radiation observed $2,019 R_J$ downstream from Jupiter⁴, where R_J is the radius of Jupiter. Given that the continuum radiation is confined to the tail cavity by the surrounding high-density solar wind close to Jupiter and also given the geometry of the observations presented in Fig. 1, it is clear that the emissions would have to propagate down the jovian tail to a point where the waves are at a frequency greater than the surrounding solar wind plasma frequency. Having reached this 'window' they could subsequently propagate freely and possibly escape in the direction of the Voyager spacecraft.

However, a comparison of the two spectra from the outer heliosphere in Fig. 4 with that in Fig. 5 taken in Jupiter's tail suggests that the two emissions have different sources. The spectrum of trapped continuum radiation varies as $f^{-\alpha}$ where α ranges between 3 and 6 (ref. 5). The radiation in Fig. 4 clearly does not show that type of frequency dependence. Moreover, the other waveform samples from both Voyagers of the new radio emission do not deviate greatly from the form shown in Fig. 4. Hence, to identify the spectra in Fig. 4 with jovian continuum radiation, one would have to rely on the solar wind medium to reshape the spectrum. The general consistency of

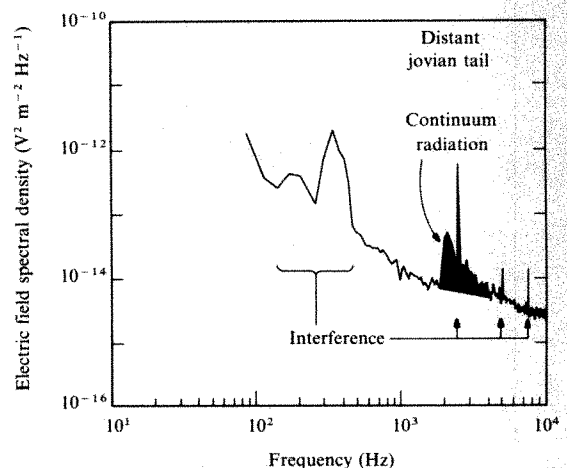


Fig. 5 A 4-s average spectrum of continuum radiation trapped in the jovian tail $2,019 R_J$ downstream from Jupiter (shaded portion) obtained by Voyager 2 on 1 February 1980 at 0016: 26 SCET. Notice that this emission is sharply peaked at lower frequencies in contrast to the emission shown in Fig. 4.

the recently obtained spectra is inconsistent with such a shaping process.

An analysis of the amplitude of the new emission also argues against a jovian source. If the continuum radiation escaping from the tail of Jupiter were to be emitted from some point along the tail (assumed to extend in the anti-solar direction from Jupiter), one would expect to see an approximate R^{-2} dependence in the intensity of the emission from the exit 'window'. From Fig. 1, we see that Voyager 1 and 2 are about 14.8 and 6.4 AU from the jovian tail axis, respectively; hence, one would expect a signal more than a factor of 5 times larger at Voyager 2 than at Voyager 1, but waveform samples taken on both spacecraft on 3 November (shown in Fig. 4) indicate that the spectral density at the peak of the emission band is nearly identical at both spacecraft. (We have used a calibration technique similar to that used by W.S.K. *et al.*⁵ which avoids the Voyager 2 calibration uncertainty.) Also, the intensity of the Voyager 1 spectrum obtained at 18 AU is only a factor of about 3 less than that obtained only 2,019 R_J (~ 1 AU) downstream from Jupiter. Hence, it seems difficult to understand the emission as jovian in origin on the basis of intensity.

We have also performed a power spectrum analysis on the data presented in Fig. 2 to look for variations near the rotation period of Jupiter since some of the radiation detected in the distant jovian tail shows amplitude fluctuations with a period of about 10 h (ref. 6). No periodicity near 10 h was detected.

Thus, while Jupiter initially seems a likely source for the emission on the basis of the frequency of the emission, the spectral shape, the lack of spatial variation in amplitude, and the lack of 10-h periodicities cast serious doubts on this interpretation. Below, we examine other possibilities for the source of the emission.

Continuum radiation trapped within Saturn's magnetosphere has also been detected⁵. Although the position of Voyager 1 might seem to be ideal to detect such saturnian radiation when one examines only the ecliptic plane projection in Fig. 1b, Fig. 1a shows that the spacecraft was actually 8 AU north of the ecliptic; hence, it is difficult to understand how any radiation from Saturn could propagate to either of the spacecraft. Moreover, as with Jupiter, the saturnian continuum radiation spectrum does not match the spectra in Fig. 4. In addition, the saturnian radiation is even weaker than Jupiter's, thus, we conclude that Saturn is not a likely source for the newly detected radio emission.

Uranus is another possible source for the radiation as we do not know what radio emissions from Uranus might look like. Using observations of the Earth, Jupiter, and Saturn as guides, the upstream planetary radio emission which most closely resembles the new emission is escaping continuum radiation from the Earth^{7,8} and Jupiter⁹. The escaping continuum radiation from those planets is extremely weak, however, and even if Uranus had similar emissions, they could not be detected by either Voyager spacecraft at their present distances unless the source was extremely bright. Other types of planetary radio emissions are intrinsically more intense than the continuum radiation; their dynamic spectra, however, are more highly structured than the emissions reported here. Nevertheless, we cannot rule out Uranus as a source of the radio noise reported above until the Voyager 2 encounter with that planet in 1986, although, we now feel that Uranus is not a likely source.

It is possible that the new radio emission detected by Voyagers 1 and 2 is generated outside the heliosphere. With our lack of information about the region, particularly at such low frequencies, any number of sources might be contemplated. Once such source is a fast pulsar which might emit enough energy at low harmonics of its pulsing rate to be detected in the frequency range of a few kHz. However, we suggest that the broad bandwidth of the newly detected signal is sufficient to rule out pulsars as the source. In addition, the power flux of the band shown in Fig. 4a integrated over the 1-kHz bandwidth is of the order of 10^{-14} W m⁻². The radio luminosity of the Crab Nebula integrated over a bandwidth of 10^8 – 10^9 Hz is about 10^{31} erg s⁻¹

(ref. 10), hence, at a distance of ~ 1 kpc this corresponds to 10^{-16} W m⁻², much less than the value observed by the Voyager spacecraft. (An equivalent, but alternate comparison, is to calculate the power spectral density from the Crab assuming the 10^{31} erg s⁻¹ is emitted in a 1-kHz bandwidth. This would be about 10^{-19} W m⁻² Hz⁻¹ compared with a value of 10^{-17} W m⁻² Hz⁻¹ observed by Voyager.) It is interesting to think of what other sources of radio noise lie beyond the Solar System, but perhaps fruitless to speculate on them at this point.

We have considered one other possibility for the source of the radio emission: the heliopause, itself. It is reasonable to assume that there is a supersonic shock marking the heliopause (sometimes referred to as the terminal shock) and shocks are known to be the source of weak, relatively narrowbanded radio noise. Specifically, Dunckel¹¹, Gurnett¹² and Hoang *et al.*¹³ have all reported radio emissions at $2f_p$ emanating from the vicinity of the Earth's bow shock and a similar phenomenon has been reported near an interplanetary shock⁴. Theories for the emission at $2f_p$ have been offered by Fung *et al.*¹⁴ and Cairns and Melrose¹⁵. The fact that this type of emission is quite weak is countered by the large source size of the heliopause and resulting large solid angle as seen by an observer in the outer heliosphere. The smoothly varying temporal character and bandwidth of the new emission are consistent with the $2f_p$ model.

If, indeed, the heliopause is generating the radio emission at $2f_p$, some interesting statements can be made concerning the size of the heliosphere. This scenario implies that the plasma frequency at the heliopause is half of 3 kHz, or 1.5 kHz; n_e at the heliopause is then ~ 0.03 cm⁻³. One can expect a maximum jump in the density at the shock of a factor of 4 (and we also assume the interstellar density is higher than just inside the heliopause), meaning the density just inside the heliopause could be as low as 0.008 cm⁻³. This corresponds to a plasma frequency of about 780 Hz. Because the density in the solar wind falls as $1/R^2$ (assuming constant solar wind speed) and the plasma frequency is proportional to $\sqrt{n_e}$, the plasma frequency varies as $1/R$. Given that f_p is ~ 2 kHz at 18 AU (using Fig. 3) and that it drops to 780 Hz at the heliopause, it is easy to solve for the radial distance of the heliopause, that is ~ 46 AU.

Obviously, the reasoning above assumes no variations in the solar wind density as a function of time and is subject to several simplifying assumptions. Also, if one assumes the density in the interstellar medium is less than just inside the heliopause, the minimum f_p in the heliosphere is 1.5 kHz and Voyager 1 is very close to that point. Taking the value of 46 AU (with liberal error bars), however, we can compare it with other predictions of the distance to the heliopause. One popular method is to interpret the cosmic ray gradient to arrive at a distance to the boundary. One such prediction of >65 AU by Webber and Lockwood¹⁶ was based primarily on Pioneer 10 observations. As Voyager 1 is travelling more or less antiparallel to the interstellar wind flow and Pioneer 10 is headed in nearly the opposite direction, it is commonly assumed that the heliopause will be encountered at smaller heliocentric radial distances by Voyager 1, hence, 46 AU does not seem to be unreasonable. Voyager 1 will be at a distance of about 46 AU in 1991 and perhaps only then will we be certain of the source of this new radio emission.

Conclusion

Voyager 1 and 2 observations of a very weak signal in the range 2–3 kHz in the outer heliosphere at distances of 13–19 AU appear to be evidence of a newly discovered radio emission. While the frequency of the radio emission is similar to continuum radiation in the distant jovian tail, other observations are inconsistent with Jupiter as a source. One other possible candidate for the emission source is $2f_p$ radiation from the heliopause. This is a most exciting possibility, because if this is the case, these observations are our first glimpse of the edge of the heliosphere.

We thank J. W. Belcher for information on the electron density observed by the Voyager 2 plasma instrument during some of the events used in this study. We also acknowledge the program-

ming support of L. J. Granroth. Research at The University of Iowa was supported by NASA through contract 954013 with the Jet Propulsion Laboratory and grant NAGW-386 with NASA Headquarters. Research at TRW was supported by NASA

through contract 954012 with the Jet Propulsion Laboratory. The research described above was performed in part at the Jet Propulsion Laboratory, California Institute of Technology and was jointly sponsored by The University of Iowa and NASA.

Received 14 May; accepted 22 August 1984.

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A third type of murine T-cell receptor gene

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By subtractive cDNA hybridizations, we have isolated a new species of T-cell receptor cDNA clone whose predicted amino acid sequence has homology to variable, constant, joining and diversity segments of immunoglobulins and T-cell receptors. The corresponding genomic sequence is also rearranged in several T-cell DNAs. The four potential N-linked glycosylation sites, frequency of expression and predicted molecular weight (27,800) of this molecule make it a likely candidate for the α -chain of the T-cell receptor. Expression data also indicate that this gene may be activated at a later stage of T-cell differentiation than the β -chain.

RECOGNITION of foreign entities by T cells is strikingly similar to that of B cells in the large number of specificities that are engendered and in the fixation in particular cell lineages of these specificities (reviewed in ref. 1). Unlike the mediators of B-cell recognition (immunoglobulins), however, T-cell receptors seem able to 'recognize' antigen only in conjunction with self-major histocompatibility complex (MHC) determinants. Cytotoxic T cells (T_c) typically require class I antigenic determinants ($H-2$)²⁻⁴ and helper T cells (T_H) require class II (Ia) determinants⁵. The affinity for self-MHC seems to be 'learned' in some way in the thymus^{6,7} before exposure to antigen and apparently allows T cells to focus on abnormalities of the organism's own cells. In addition, helper T cells up-regulate and suppressor T cells down-regulate B cells and other immune system-related responses.

Uncertainties concerning the biochemical and genetic nature of the T-cell receptor molecule(s) has been a major stumbling block in understanding the phenomena described above. Continual progress, especially in the past few years, has begun to provide some answers. In particular, T-cell cloning methodologies using either long-term lines or hybridomas¹ have allowed a better definition of the antigen-MHC reaction phenomena and have also provided large quantities of homogeneous cell populations of a single, defined specificity. This has also led to the development of clone-specific (anti-idiotypic) antisera⁸ and monoclonal antibodies⁹⁻¹³ which can either block or stimulate the response of the target cells to antigen¹⁰⁻¹³. These 'clonotypic' monoclonal antibodies were used successfully to precipitate the putative T-cell receptor molecule itself, an 80,000-90,000 dalton molecular weight (80-90K) sulphhydryl-linked heterodimer⁹⁻¹³ which on reduction yields α - and β -subunits of 40-50K each. These subunits seem to have both variable and constant fragments in tryptic peptide maps^{14,15}. Recently, cDNA clones encoding one chain of the T-cell receptor were isolated in mouse^{16,17} and man¹⁸. N-terminal

amino acid sequencing established that these clones encode the β -chain¹⁹ of the T-cell receptor heterodimer. The β -chain cDNA clones encode a 32K protein having variable, constant and joining (J) region elements remarkably similar to those of immunoglobulins^{17,18}. More recently, D (diversity) elements have also been shown to play a part²⁰⁻²³ in the formation of the

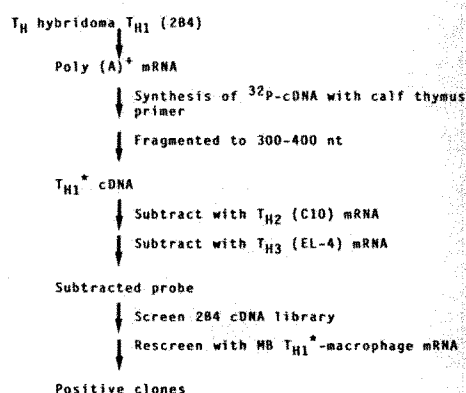


Fig. 1 cDNA subtractions and screening strategy. Calf thymus DNA was used to synthesize randomly primed ³²P-labelled cDNA by standard procedures⁴⁷ from poly(A)⁺ cytoplasmic 2B4 mRNA. The cDNA was initially 700 nucleotides (nt) average length and was allowed to fragment by autoradiolysis to ~300 nt long over a 2-week period. The subtractive hybridization was carried out by procedures described previously⁴². The ³²P-cDNA was first hybridized and hydroxyapatite-selected with the T_H hybridoma C10 mRNA, then with the T_H -like lymphoma line, EL-4. The twice-subtracted probe was then hybridized to a filter of 2B4 cDNA library in the vector λ gt10 as described elsewhere²⁰. Approximately 20,000 plaques were screened. Seven positive plaques were picked and rescreened with a probe of oligo(dT)-primed cDNA made of membrane-bound polysomal mRNA from 2B4, subtracted with mRNA from the P388D₁ macrophage line (MBT₁-Mac).

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variable-region exon of this gene. The mechanism of rearrangement seems very similar to that of immunoglobulins²⁰⁻²², the only major difference being the extreme diversity and apparently accelerated rate of divergence of the different variable regions, apparently due to the presence of additional hypervariable regions in the molecule²⁴. These data have helped to elucidate the problem of diversity and clonal fixation of specificities by direct analogy with immunoglobulin.

The other questions specific to T-cell recognition are more difficult and require knowledge (and possession) of the gene encoding the second chain of the heterodimer (α). An apparent

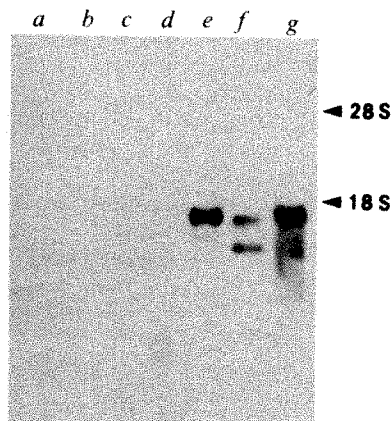


Fig. 2 T cell-specific expression of TT11. The TT11 cDNA clone was labelled by nick-translation⁴⁵ and hybridized to a Northern blot⁴⁵ containing a panel of mRNAs as follows: *a*, Bal 17, B-cell lymphoma; *b*, M104E, plasmacytoma; *c*, 3T3, fibroblast line; *d*, P388D₁, macrophage line; *e*, 2B4; *f*, EL-4; *g*, BW5147. All are poly(A)⁺ cytoplasmic RNAs prepared by standard procedures⁴⁵.

answer in this regard was obtained by Saito *et al.*²⁵, who used subtractive cDNA selection methodologies¹⁶ to obtain a second immunoglobulin-like, T cell-specific cDNA clone (HDS4 and related species) which is rearranged in T_c DNAs and has elements homologous to variable, constant and *J* regions. Although this gene may encode one of the chains of the T-cell receptor for murine cytotoxic T cells, it seems unlikely that it also encodes the α -chain for the murine helper T cells for the following reasons: (1) there are no N-linked glycosylation sites (N-X-S) in the amino acid sequences predicted for the HDS4 series

clones, whereas three N-linked additions have been found in α -chains from three different murine helper T-cell lines²⁶⁻²⁸, including 2B4 (ref. 26), the subject of this report. (2) The HDS4 gene is not expressed in many T_H-cell lines (our results and S. Tonegawa, personal communication). That T_H and T_c cells use the same β -chain locus has been established^{25,29-31} by molecular genetic analysis. (3) A consistently low level of expression has been seen for this gene with respect to β -chains, that is, 1% of the level of β -chain expression in thymus and 2% of the level in spleen cDNA libraries (this paper), and $\leq 10\%$ of the β -chain level in some T_c-cell libraries (Z. Eshhar, personal communication). Although differences in translational efficiency or stability are possible, all known multi-chain molecules have mRNA species present in approximately equal amounts (see below).

In view of these questions, we generated a new cDNA probe designed to detect variable regions which might differ between various T-cell lines, and used this to screen a T_H cDNA library from the cytochrome *c*-specific hybridoma, 2B4 (ref. 12). One of the cDNA clones obtained in the screen, TT11, appears to encode a protein with many of the expected features of an α -chain and is expressed in both T_H and T_c cells. If this is confirmed to be the case, its characterization will identify the missing portion of the T-cell receptor molecule. However, this result also raises the interesting question of why there are three (or more) immunoglobulin-like chains in at least some T cells.

Isolation of cDNA clones

As peptide mapping has shown that the α -chains of the T-cell receptor molecules isolated from T_H cells are as diverse as the β -chains¹⁵, and given the distinct immunoglobulin-like structure of the β -chain of the T-cell receptor^{17,18} and the likelihood that the α -chain is encoded by a similar molecule, it seemed feasible to prepare a variable (*V*)-region-specific subtracted cDNA probe between T cells of differing specificities. This was achieved as shown in Fig. 1, where randomly primed, labelled cDNA⁴⁷ from the mRNA of the helper hybridoma, 2B4 (ref. 12), was synthesized; this probe was allowed to fragment by autoradiolysis to an average size of 300–400 nucleotides and then subtracted, first with C10 mRNA, then with EL-4 mRNA. The resultant probe should be enriched in 2B4 *V*-region-specific sequences, and was then used to screen a 2B4 cDNA library made in the vector λ gt10²⁰. The use of calf thymus DNA as a random primer gives fairly equal reverse transcription over the entire length of each mRNA species, thus eliminating the 3'-end bias of oligo(dT) priming. The size of 300–400 nucleotides is optimal in that it is large enough to give good reactivity in hybridizations,

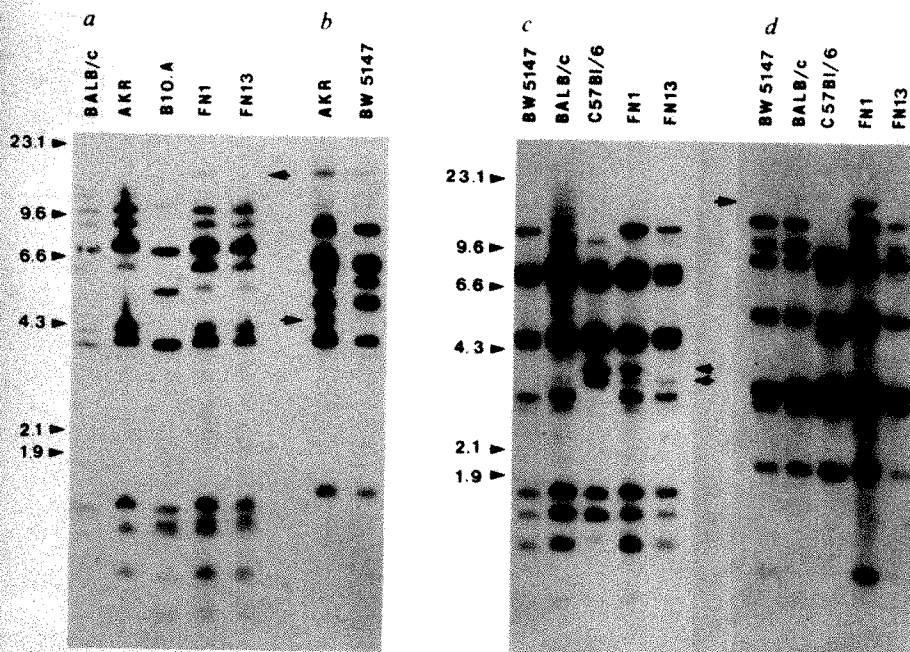


Fig. 3 Rearrangement of the TT11 gene. Genomic DNAs from the livers of different mouse strains, various T-cell lines and hybrids of the B-cell lymphoma L10A, were digested with: *a*, HindIII; *b*, EcoRV; *c*, XbaI; *d*, BglII and electrophoresed through 0.7% agarose, blotted onto nitrocellulose and hybridized by standard methods⁴⁵ to a probe from the 5' half of TT11 (RI-RV; see Fig. 4). Arrows indicate altered bands, arrowheads indicate molecular weight markers. In the HindIII digest (*a*) the B10.A pattern is identical to that of the true parental, C57Bl/6.

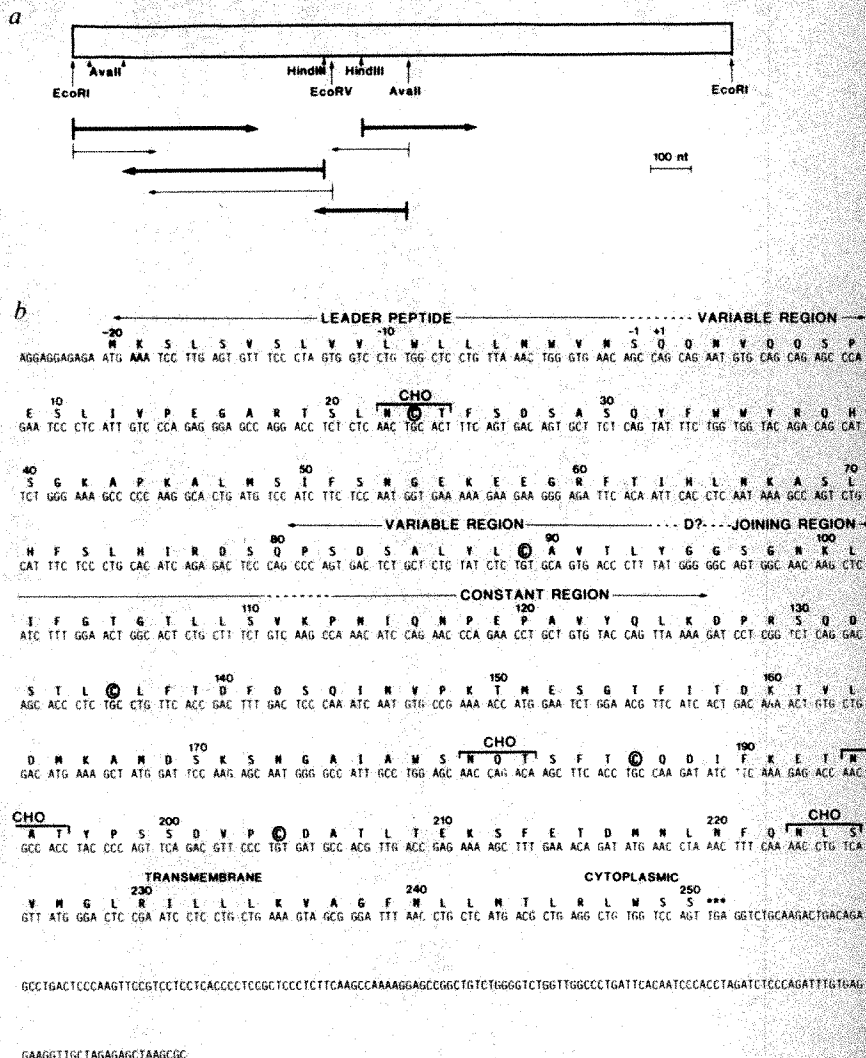


Fig. 4 *a*, Sequencing strategy for the coding portion of TT11 cDNA clones. The *EcoRI* sites are generated by the RI linkers. Thin lines indicate 5'-end labelling with polynucleotide kinase and thick lines indicate 3'-end labelling with the large (Klenow) fragment of DNA polymerase I. *b*, The determined nucleotide sequence and the predicted amino acid sequence. The numbers above the amino acid sequence designate the residue positions. The leader, variable, possible D, joining, constant, transmembrane and cytoplasmic regions are indicated although the exact boundaries are somewhat ambiguous. Cysteines are circled. Potential signal sequences for N-linked glycosylation are indicated by CHO.

yet, as it is approximately the same size as immunoglobulin and T-cell receptor β -chain *V* regions, at least some fractions of the species will not have *C* region-hybridizable sequences. One complication in such a screening protocol is the fact that even phenotypically identical cell lines and hybridomas appear to differ by a small but significant fraction of their gene expression (0.2–0.4%; M.M.D., S. M. Hedrick and W. E. Paul, unpublished observation), perhaps through random gene loss or activation. As β -chain mRNA expression is generally much lower than this in hybridomas (0.02%)²⁰, it was necessary to subtract the probe with two separate mRNAs (as shown in Fig. 1) from T cells which do not share the same specificity. C10 and EL-4 have distinct T_H phenotypes similar to 2B4 but are not cytochrome *c*-reactive. There is, of course, some uncertainty in constructing such strategies (see Fig. 1) because even with cells of differing specificity, the same or cross-reactive *V* regions may be expressed. Seven positive plaques obtained in this primary screen were rescreened with another cDNA probe made from the membrane-bound polysomal RNA of 2B4 subtracted with RNA from the macrophage line, P388D₁ (ref. 32). This provides an additional selection for T-cell receptor molecules^{16,25}. Three of the seven plaques were positive with the MBT_H-Mac probe (using the convention of ref. 16) and two of these three cross-hybridized with each other. The one with the largest insert, TT11, was analysed further.

Pattern of expression and rearrangement

Figure 2 shows that, on Northern blot analysis, TT11 is T-cell-specific, as it is not expressed in the B-cell lymphoma (Bal 17), a plasma cell line (M104e), a macrophage line

(P388D₁) or a fibroblast line (3T3). It is expressed in all T cells currently surveyed, including the two, used in the T^H -T subtractions: EL-4 (Fig. 2) and C10 (data not shown), as well as in three T_c -cell lines (T.L. *et al.*, work in progress). It is interesting to note the two distinct bands at 1.8 and 1.3 kilobases (kb) in the EL-4 lane; the possible significance of this with respect to *D*-like regions in this molecule will be discussed below.

The gene(s) encoding the TT11 cDNA clone show distinct signs of rearrangement (Fig. 3), characteristic of all the immunoglobulin-like B-cell-(IgH, Ig κ and Ig λ)³³ and T-cell-(β , HDS4)^{16,25} derived genes. Arrows in Fig. 3 indicate bands which have changed position with respect to the parental genomes in the T-cell DNAs. FN1 and FN13 are keyhole-limpet haemocyanin (KLH)-reactive T_H hybridomas (referred to in ref. 34 as FN1-18 and FN13-21) derived, respectively, from BALB/c $\times$ C57B/6 strain mice and the AKR strain thymoma line, BW5147. In Fig. 3a, a new band appears in a *HindIII* digest of FN1 (arrow) with respect to parental DNAs. In Fig. 3b, one band in an *EcoRV* digest of AKR liver DNA disappears in BW5147, and an *EcoRI* digest shows a new band appearing in BW compared with the parental liver DNA (data not shown). In Fig. 3c, the arrows indicate two bands which are polymorphic for an *XbaI* digest of C57B/6 DNA; both of these bands are present in the FN1 hybrid but only one occurs in FN13, which could only be due to a rearrangement or partial deletion of the chromosome. Figure 3d shows a new band in FN1 versus the parental DNA samples in a *BglII* digest. Although no digest shows evidence of rearrangement in all T-cell DNAs, these results provide sufficient indication of such events to suggest that TT11 is another T-cell receptor-like gene.

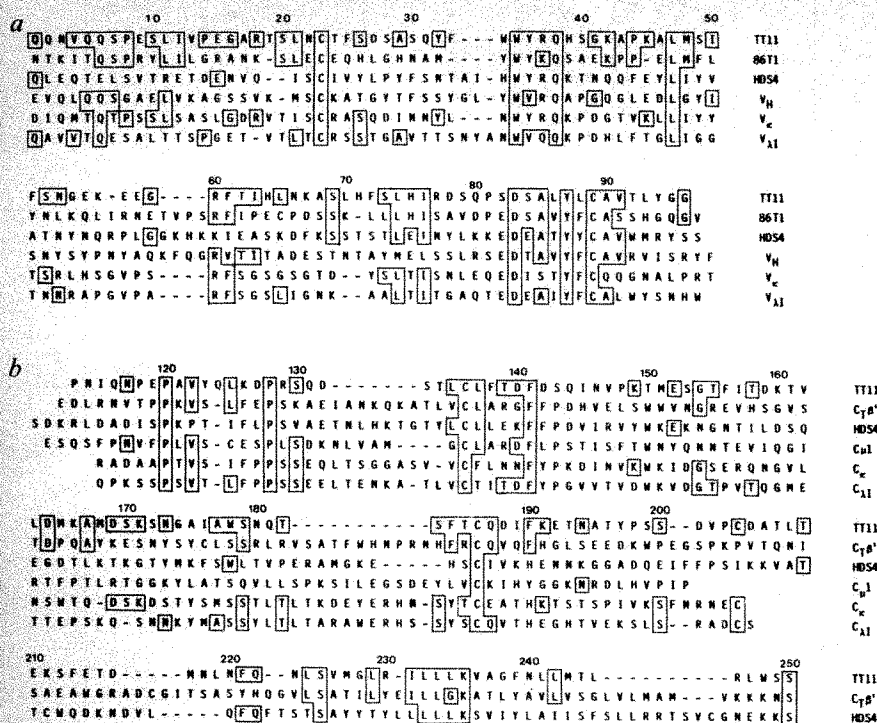


Fig. 5 Similarities of the TT11 amino acid sequence to variable (a) and constant (b) regions of a murine T-cell receptor β -chain 86-T1, HDS4 and immunoglobulin. Similarities are boxed, with gaps represented by dashes (-).

TT11 homology with immunoglobulins

The TT11 cDNA clone was partially sequenced by the procedure of Maxam and Gilbert³⁵ using the strategy shown in Fig. 4a; the sequence is shown in Fig. 4b. The clone was oriented by a poly(A) stretch (~150 nucleotides) at the 3' end and sequencing of the 5' half revealed a long open reading frame of 810 nucleotides, with an initiation codon (ATG) within the first 12 nucleotides (Fig. 4b). The sequence has regions similar to the immunoglobulin leader, variable regions, J region and constant region, as is the case also for the T-cell receptor β -chain^{17,18} and the HDS4 gene clone of Saito *et al.* Also, as in the latter two T cell-specific genes, TT11 has an extra cysteine outside the putative intra-domain cysteines in the constant region (which probably bonds to another chain), followed by a transmembrane portion and a potential cytoplasmic region, all of which are encoded as separate exons in the β -chain genes^{36,37}. There are four potential N-linked glycosylation sites, as indicated in Fig. 4b, similar to the four or five found in different β -chain sequences^{17,20}. Probably only three of the four potential sites are available for glycosylation as the most carboxy-terminal one is embedded in the putative transmembrane region (Fig. 4b).

The exact position at which the murine T-cell receptor protein is processed is unknown, but by analogy with all of the immunoglobulins (H, λ , κ), it should occur just before the glutamine at position +1 (see Fig. 4b). This is consistent with the finding that murine α -chains are blocked at their N-terminus (J. Allison, personal communication, and L. Samelson, personal communication). Alternatively, based on the N-terminal amino acid sequence of the human β -chain from the REX T-cell line¹⁹, the processing point would be just before the asparagine at position +3. In the first case, the molecular weight would be 27.8K, in the second slightly less; this agrees well with the data of Allison and colleagues²⁷ who obtained a molecular weight of 27K from a murine α -chain stripped of N-linked sugars with endoglycosidase F.

Comparisons with other putative T-cell receptor molecules and immunoglobulin V, C and J regions are shown in Figs 5 and 6, respectively. While the overall homology of the putative V and C regions with their immunoglobulin counterparts (10–26%) is relatively low, many of the conserved residues found

in all of five known immunoglobulin-like genes are present, particularly in the V region (Fig. 5) and J-region elements (Fig. 6). The cysteines in the TT11 variable region are 65 amino acids apart, which is identical to the spacing in the light chains, and the sequence WYRQ starting at residue 35 and DSA-Y-CAV at residues 83–91 are also highly conserved in most immunoglobulin and T-cell receptor V regions. As with the β -chain and HDS4, the putative J region is the most highly conserved portion, with 7 out of 16 residues homologous with the β -chain consensus sequence³⁶ and 9 out of 16 the same as J_T3 (ref. 36). Whereas the constant region has the least homology of any of these (10–15%), its overall structure is very similar to that of β -chain and HDS4. Somewhat more homology (up to 259) may be obtained in the constant region by using the cysteine at position 204 as the intradomain linkage, but this complicates matters with respect to the presumptive β -chain linkage.

Also characteristic of the T-cell receptor sequences, and shared with TT11, is the sequence ILLK in the putative transmembrane region. The conservation of a charged amino acid (lysine or K) in a transmembrane region is unusual and is not found in other members of the immunoglobulin superfamily; its presence in all three of the putative T-cell receptor molecules may indicate that some unusual multi-chain structure is formed with other molecules in the membrane.

Table 1 Frequencies of T-cell receptor expression

	$C_T\beta$	TT11	HDS4
Thymus	0.2%	0.01%	0.002%
Con A stimulated spleen	0.1%	0.03%	0.002%
2B4	0.02%	0.03%	<0.001%

Approximately 200,000 phage from each library were screened by standard procedures in duplicate. The $C_T\beta$ probe was 86T5 (ref. 17), a DJC²²-containing cDNA clone; the HDS4 probe was a 150-nucleotide synthetic polymer corresponding to the constant region of HDS4 from a.a. 120 → a.a. 180. The former was labelled by nick-translation and the latter by a fill-in reaction with Klenow polymerase. The TT11 probe was labelled by nick-translation.

S	G	N	K	L	I	F	G	T	G	T	L	S	V	K		TT11
-	-	-	-	L	V	F	G	-	G	T	R	L	T	V	L	J _β consensus
S	G	N	L	T	L	V	F	G	E	G	S	R	L	I	V	J _{T3}
G	F	H	K	V	F	A	E	G	T	K	L	I	V	I		HDS4
-	-	Y	F	D	Y	M	G	Q	G	T	V	T	V	S	S	J _H consensus
-	-	T	F	G	G	G	T	K	L	E	I	K	R			J _K consensus
W	V	F	G	G	T	K	V	T	V	L	G					J _λ consensus

Fig. 6 Amino acid homologies among the putative J region of TT11 and the J-region sequences of a murine T-cell receptor β -chain, J_{T3}, HDS4 and immunoglobulins. Similarities are boxed, with variable residues represented by dashes (-).

Possible D regions

Another interesting feature of the TT11 sequence analysis is the strong indication that D regions are present in this molecule just as in the β -chain of the T-cell receptor²⁰⁻²³ and immunoglobulin heavy-chain genes³³. In particular, just 5' to the SGN amino acid sequence of the putative J region (which marks the 5' border of J_{T3} in the β -gene complex), there is the nucleotide sequence GGGGG. This sequence occurs at the point where a D-region element would begin in the β -chain and is characteristic of the β -gene D regions (7 out of 14 contain runs of between 3 and 7 Gs on their 3' side)³⁸ as well as many immunoglobulin heavy-chain D regions³³. Although postulated to be the result of enzymatic addition in immunoglobulins⁴⁸, at least in some β -chain and Igh D regions, these poly(G) sequences are present in germ-line sequences^{22,23}. None of the immunoglobulin light-chain V-region genes sequenced has such poly(G) sequences before the J element junction³⁹. The Northern blot data of Fig. 2, where two bands are clearly visible in EL-4 (and other T_H lines, data not shown), also support the existence of independently assorting D gene segments. This two-band pattern is characteristic of the DJC transcript of the β -chain which is 300 nucleotides shorter than the VDJC transcript^{22,23}. Taken together, these data suggest that the TT11 gene will have D segments, adding to the potential diversity.

Frequency of expression

One characteristic of mRNA species which contributes to a multimeric structure is that they appear to be present in equal or nearly equal percentages in the steady-state mRNA population in cases that are known. Immunoglobulin heavy and light chains⁴⁰, Ia α - and β -subunits⁴¹, H-2 and β_2 -microglobulin⁴², and α - and β -globins⁴³ all follow this rule to within a factor of ≈ 2 . Although differential translation efficiencies or turnover rates could conceivably produce large differences in subunit mRNA frequencies, they do not seem to do so in these cases. To compare the steady-state mRNA frequencies in a relatively quantitative manner, we surveyed thymocyte, concanavalin A (Con A)-stimulated spleen and 2B4 cDNA libraries with TT11, HDS4 and C_T β probes. The results (Table 1) indicate that TT11 and C_T β are present in fairly similar frequencies (1:1-1:3) in the 2B4 and Con A spleen libraries, whereas HDS4 is much rarer, being present in $\frac{1}{50}$ th the amount of β -chain in the Con A spleen library and absent from the 2B4 library. This equivalence in frequency, together with the other data presented here, makes TT11 a more attractive candidate for the α -chain of the receptor in most T cells. Also of interest from the data in Table 1 is the change in the ratio of TT11 to β -chain in immature versus mature T cells. Specifically, the TT11/ β -chain ratio changes from 1:20 in thymocytes to 1:3 in Con A-stimulated spleen cells, suggesting that TT11 gene expression (and perhaps rearrangement) may occur after that of the β -chain, analogous to light-chain immunoglobulin expression following that of the heavy chain in B cells³³.

How many T-cell receptor gene families?

Regardless of whether TT11 or HDS4, or neither, encodes the T-cell receptor α -chain, there is already an overabundance by

at least one of immunoglobulin-like T cell-specific genes which rearrange in some types of T-cells. The reason for this cannot be explained based on the present biochemical evidence, and may involve variations of a two-receptor model of T-cell recognition⁴⁹. Although the strict 'two-receptor' model for MHC-antigen co-recognition has been excluded by several convincing experiments^{44,50}, there exists the possibility that in at least some circumstances an alternative heterodimer might be able to form for some purpose related to this event.

We thank Dr J. Casey for the gift of random primer DNA, Dr C. Benoist for the thymocyte and spleen cDNA libraries, Dr C. Bona and colleagues for the FSN-1 and FSN-13 DNAs, Dr E. Ruley for 3T3 mRNA, Dr K. Arai and colleagues for the HDS4 probe, Dr J. Rothbard for the molecular weight calculation and G. Samuel and K. Redman for secretarial assistance. D.M.B. was supported in part by a fellowship from the Lupus Foundation, T.L. is a fellow of the Damon Runyon-Walter Winchell Cancer Fund and D.C. was a fellow of the Leukemia Society of America. This work was supported by grants to M.M.D. from NIH and the American Cancer Society.

Note added in proof: Amino acid sequencing of a human T-cell receptor α -chain (N. Jones and J. L. Strominger, personal communication) suggests that TT11 is the murine homologue. Also, most of the 8-10 bands seen on Southern blot (Fig. 3) with a TT11 probe hybridize to the variable portion of the molecule. This contrasts with results obtained with V _{β} probes²⁴ and suggests that V _{α} may be immunoglobulin-like.

Received 9 September; accepted 1 October 1984.

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A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes

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In addition to the two previously identified genes rearranged and expressed in a cytotoxic T-lymphocyte clone, we have identified a third gene that is also rearranged and expressed in the same clone. This new gene shows clonal diversity, codes for a polypeptide chain that contains immunoglobulin-like variable and constant domains, carries potential N-glycosylation sites and is a particularly attractive candidate for the gene that encodes the α -subunit of the heterodimeric antigen receptor of this T-cell clone.

IN spite of its central roles in both regulatory and effector phases of the vertebrate cellular immune system, the chemical nature of the T-cell antigen receptor has been elusive¹. A particularly intriguing question about this receptor concerns the molecular basis for its dual recognition of antigens in conjunction with self major histocompatibility complex (MHC) products²⁻⁴. Unlike immunoglobulins, which recognize and bind free antigens, the T-cell antigen receptors characteristically recognize cell-bound antigens in the specific molecular context of self-MHC molecules. Recently, several groups have succeeded in making antisera and monoclonal antibodies that are specific for individual T-cell clones⁵⁻⁷ and which presumably recognize clone-specific determinants on the T-cell antigen receptors. The receptor thus characterized is a heterodimeric glycoprotein of apparent relative molecular mass (M_r) ~90,000, consisting of an α -subunit (M_r 42,000–44,000) and a β -subunit (M_r 42,000–44,000) held together by an inter-chain disulphide bond(s). Peptide fingerprint analyses of α - and β -chains from several T-cell lines suggest that both subunits are composed of variable (V) and constant (C) regions⁸⁻¹⁰.

During the past year, three groups have isolated cDNA clones [YT35 (ref. 11), TM86 (ref. 12), pHDS11 (ref. 13) and pHDS4/203 (ref. 13)] that probably correspond to T-cell receptor genes. As YT35, TM86 and pHDS11 from a human T-cell tumour, a mouse T helper hybridoma and a mouse cytotoxic T lymphocyte (CTL) clone, respectively, are all extensively homologous to each other at the 3' half, it was concluded that they correspond to the same gene. Recently, partial amino acid sequences determined by direct analysis of the β -chain of a human T-cell receptor showed that YT35, TM86 and pHDS11 represent the gene for this subunit¹⁴. Although there is no extensive nucleotide homology between the fourth clone, pHDS4/203, and the other three cDNA clones, all share several common properties. (1) They are expressed in T cells but not in B cells. (2) The corresponding genes are rearranged in T cells and not in B cells. (3) In the encoded proteins there are distinctive regions that, proceeding from amino- to carboxy-terminus, correspond to a signal peptide, two immunoglobulin-like domains, a transmembrane peptide rich in hydrophobic amino acids and a short cytoplasmic peptide. (4) Their deduced amino acid sequences have low but significant homology to those of immunoglobulin chains. (5) Each gene is composed of separate V, J and C gene segments¹⁵⁻¹⁸ (in the cases of YT35 and TM86, the corresponding genes have also been shown to have D segments^{19,20}). (6) Their V, (D) and J segments have characteristic signal sequences for gene rearrangement (heptamer and nonamer separated by either 12 or 23 base pairs (bp); refs 15, 16, 18–20 and A. Hayday *et al.*, unpublished data).

The cDNA clone pHDS4/203 therefore satisfied many of the

properties expected for the α -chain of the T-cell antigen receptor. However, this clone lacks sequences that correspond to sites for N-linked glycosylation and it has recently been shown that both the α -chain and the β -chain of another type of T cell, helper cells, are N-glycosylated (J. P. Allison, personal communication). Although it was possible that the α -chain of the CTL clone from which pHDS4/203 was isolated is not N-glycosylated, it also seemed possible that there is still another gene (or genes) whose properties are similarly consistent with those expected for the α -subunit of the T-cell receptor. Accord-

Table 1 T cell-specific clones from a subtracted CTL cDNA library

Group	Size of mRNA (kb)	No. of clones	Rearrangement	Representative clone
T (thy1)	2.0	19	No	pHDS1
B	0.8	19	No	
C(β)	1.4	18	Yes	pHDS11
F	1.7	13	No	
E	0.8	12	No	
D	1.7	9	Yes	pHDS58
K	1.3/1.1	9	No	
G	1.2	8	No	
A	1.5	4	Yes	pHDS4/203
P	2.3	2	No	
Q	n.d.	2	No	
I	2.2	1	No	
J	1.0	1	No	
L	1.5	1	No	
N	4.4	1	No	
R	0.8	1	Maybe	pHDS86
Total		120		

Two hybridization probes were used to screen the cDNA library. The first was the 2C cDNA prepared from the poly(A)⁺ RNA of membrane-bound polysomes by subtraction with poly(A)⁺ RNA from a B-cell lymphoma, CH-1. The second was the cDNA prepared from the total poly(A)⁺ RNA from another B-cell lymphoma A20-2J. After screening about 20,000 independent transformants, we have identified a total of 140 putative T cell-specific cDNA clones and grouped these clones on the basis of the corresponding genes, using the following procedures. First, plasmid DNA preparations from each of 20 randomly chosen cDNA clones were used as hybridization probes in RNA blotting analysis of 2C poly(A)⁺ RNA and DNA blotting analysis of 2C and embryo DNA. The hybridization patterns allowed assignment of these clones to 12 groups. Second, mouse DNA inserts were dissected from a representative cDNA clone of each group and used as probes for colony hybridization of all of the cDNA clones; the results allowed assignment of 105 clones among the 12 groups. The above procedures were repeated for some of the remaining unassigned clones.

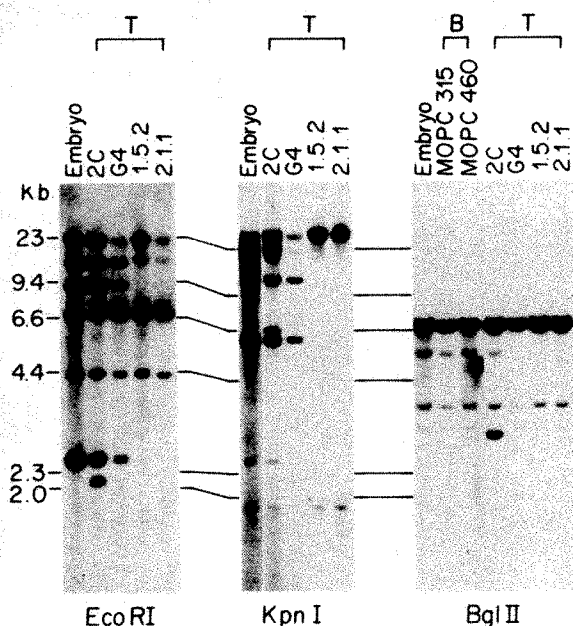


Fig. 1 Southern blot analyses²² of DNA from BALB/c embryo, myelomas MOPC315 and MOPC460 (both BALB/c-derived) and CTL lines 2C, G4, 1.5.2 and 2.1.1 (all BALB.B-derived²¹). DNA was digested with the indicated restriction enzymes, electrophoresed through 0.8% agarose gels, blotted onto nitrocellulose and hybridized with the ³²P-labelled, nick-translated insert from clone pHDS58. Hybridization was carried out at 42 °C in 50% formamide and 5 × SSC. The filters were washed at 65 °C in 0.2 × SSC. Separate experiments showed that BALB/c and BALB.B embryo patterns are indistinguishable. Numbers on the left-hand side are *M_r* markers.

ingly, we have analysed further the cDNA library from CTL clone 2C and report here the isolation of a third class of cDNA clone representing a gene whose protein product is a better candidate for the α -subunit of the T-cell $\alpha\beta$ heterodimer receptor than is pHDS4/203: the new gene is rearranged and expressed specifically in T cells, its product has the sequences expected of an integral membrane protein and it carries potential N-glycosylation sites.

Classification of cDNA clones

We reported previously¹³ construction of a 'subtracted' cDNA library from an alloreactive CTL clone (2C) specific for the product of a gene at the *D* end of the *H-2* complex²¹. We subjected this library to differential screening using two hybridization probes (Table 1). From these experiments, it was established that 120 of 140 putative T cell-specific cDNA clones originally identified, belonging to 16 different groups, are expressed specifically in CTL clone 2C and not in B cells. Table 1 lists the number of cDNA clones in each group and the size of poly(A)⁺ RNA they detected by Northern hybridization. Of the remaining 20 cDNA clones, 12 belonged to two gene groups whose expression in B cells was substantially lower than in T cells but still definitely detectable. The other eight cDNA clones contained relatively short inserts (less than 250 bp) and have not yet been characterized.

Among the 16 T cell-specific cDNA groups, we identified four whose genes seemed to be rearranged in CTL clones. Two (pHDS11 and pHDS4/203) of the four have been described previously¹³. The characteristics of the third group, represented by pHDS58, are the main subject of the present report. The fourth cDNA, represented by one member, pHDS86, hybridized to an extra DNA fragment when *Pvu*II digests of clone 2C DNA and embryo DNA were compared; however, nine other restric-

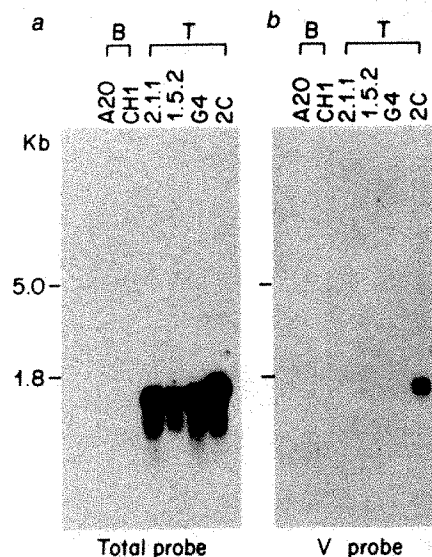


Fig. 2 RNA blot analyses²³ of poly(A)⁺ RNA from various B- and T-cell lines. Probes: *a*, total insert of pHDS58; *b*, *Hpa*II fragment containing 270 bp at the end of the pHDS58 insert (V-region probe). RNA was extracted from B-cell lymphomas A20-2J and CH1 and alloreactive (H-2^b anti-H-2^d) cytotoxic T-lymphocyte clones 2.1.1, 1.5.2, G4 and 2C. Approximately 1.5 μ g of poly(A)⁺ RNA was denatured with glyoxal and electrophoresed through a 1% agarose gel in 10 mM sodium phosphate buffer (pH 6.5). RNA was transferred to a nitrocellulose membrane and hybridized to ³²P-labelled nick-translated probe DNAs. The conditions of hybridization and washing are given in Fig. 1 legend. The filter used for hybridization with the first probe was washed by boiling at 100 °C for 5 min in H₂O and re-used for hybridization with the second probe.

tion enzymes failed to show any evidence of rearrangement (data not shown).

Gene rearrangement in T cells

Figure 1 shows the results of Southern blot analyses²² of DNA from BALB/c embryos, four CTL clones of different specificities and two myelomas that have been digested with *Eco*RI, *Kpn*I or *Bgl*II and hybridized with the insert of pHDS58. With each of the three enzymes, clone 2C DNA showed an extra fragment that was not detected in the digest of embryo DNA. DNA from the other CTL clones gave hybridization patterns which lacked several fragments detected in embryo DNA. In contrast, the hybridization patterns of DNA from the myelomas and embryos were indistinguishable. These results strongly suggest that the gene or genes corresponding to clone pHDS58 are rearranged in cytotoxic T cells but not in myelomas.

The expression of the pHDS58 genes in CTL clones and B lymphomas was examined by Northern hybridization analyses²³ of poly(A)⁺ RNA. When the whole insert of pHDS58 was used as the hybridization probe, no hybridization was detected in the two B-cell lymphomas, but poly(A)⁺ RNAs of distinct sizes were detected in all four CTL clones (Fig. 2*a*). The size of the major RNA species in 2C (1.7 kilobases, kb) was slightly greater than that in the other three CTL clones (1.6 kb). In addition, minor RNA species of about 1.2 kb were detected in all four CTL clones. When a DNA fragment containing 270 bp from the 5' end of the pHDS58 insert was used as the hybridization probe, only the 1.7-kb RNA species of 2C was detected and the other three CTL clones and the B-cell lymphomas exhibited no hybridization (Fig. 2*b*; see Fig. 3 for the region of the cDNA sequence covered by the probe). These results suggest that the 1.6–1.7-kb RNA is composed of a common 3' sequence (C region) and a CTL clone-specific 5' sequence (V region), as has been seen with immunoglobulin and the T-cell receptor β -chain.

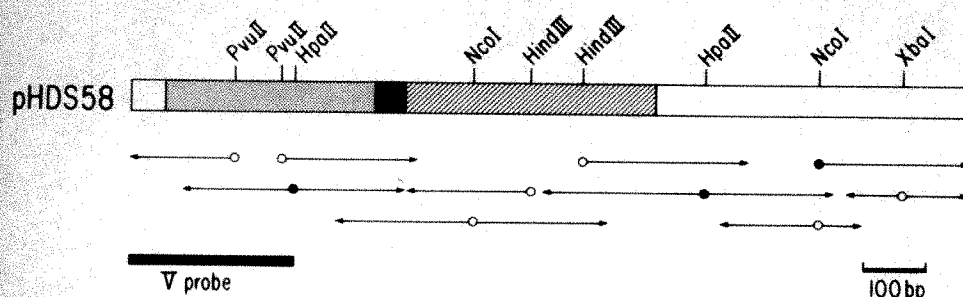


Fig. 3 Restriction map of the insert of cDNA clone pHDS58. The map was constructed by a combination of single and double restriction enzyme digestions of the plasmid DNA. The V, J, C and 5'- or 3'-untranslated regions are shown by shading, black, hatching and white, respectively. Also shown is the sequencing strategy used to determine the nucleotide sequence shown in Fig. 4. The arrows indicate the directions and the extent of sequence determination. The open and closed circles indicate that the ends of the DNA fragments labelled are 5' and 3', respectively.

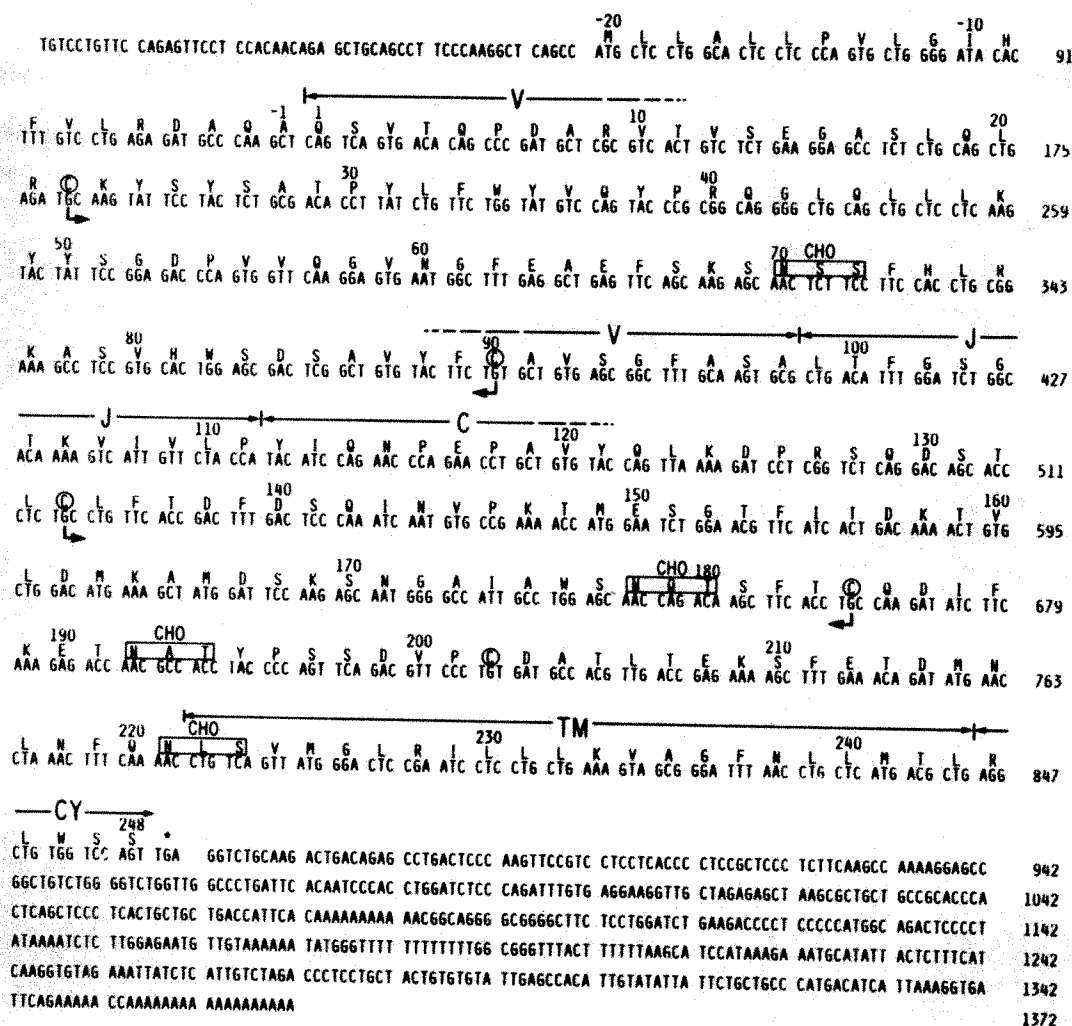


Fig. 4 The nucleotide and predicted amino acid sequences of the cDNA clone pHDS58. The nucleotide sequence was determined by the method of Maxam and Gilbert²⁴ according to the strategy shown in Fig. 3. Numbers given above the amino acid sequence designate amino acid residue positions. The numbers on the right show nucleotide positions. The V, J, C, TM (transmembrane) and CY (cytoplasmic) regions are indicated by horizontal arrows although exact boundaries are ambiguous. Cysteine residues thought to be involved in intradomain or inter-chain disulphide linkages are encircled. The potential N-glycosylation sites (N-X-S or N-X-T) are also indicated.

Nucleotide sequence

The restriction map and the sequencing strategy of the pHDS58 insert are shown in Fig. 3. The entire nucleotide sequence of 1,372 bp determined by the method of Maxam and Gilbert²⁴ is shown in Fig. 4. The longest open reading frame begins with the Met codon at nucleotide positions 56–58, extends over a stretch of 804 bp, and ends at nucleotide position 859. The amino

acid sequence corresponding to this reading frame is also shown in Fig. 4. For reasons given below, the numbering of codons or amino acid residues starts with the triplet CAG (Gln) at nucleotide positions 116–118. The amino acid sequence immediately following the Met and extending to the Gln is highly hydrophobic and reminiscent of a signal peptide²⁵. After the Gln, the sequence is significantly homologous to those of the T-cell

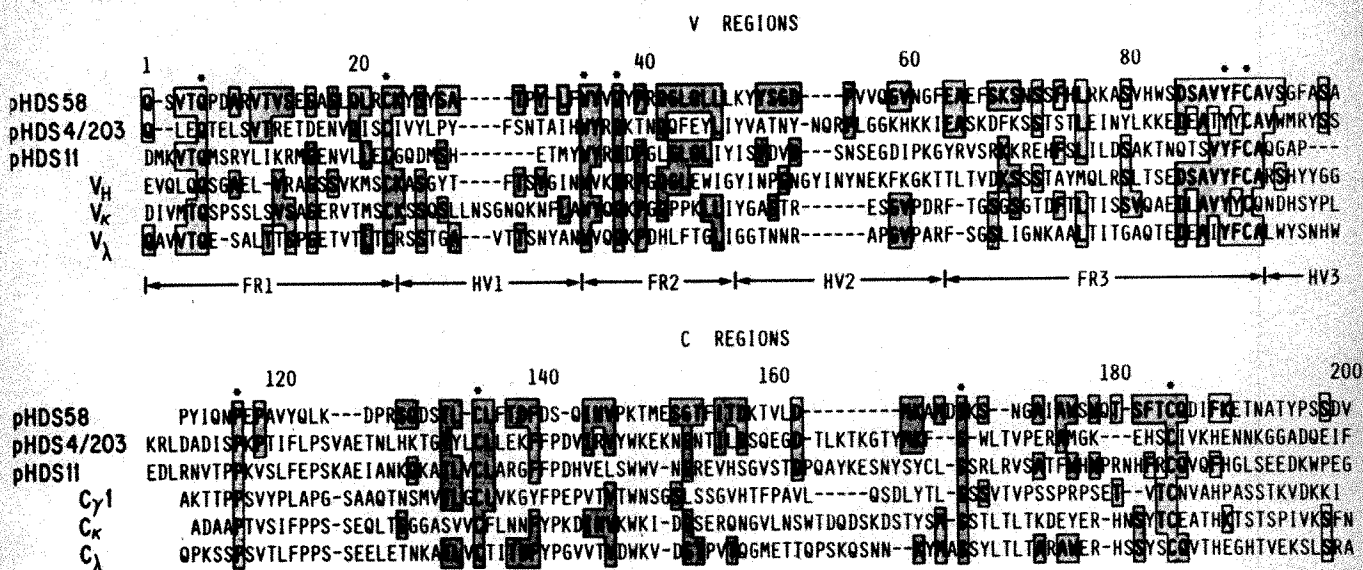


Fig. 5 Comparisons of the V and C regions of the predicted amino acid sequence of pHDS58 and the V and C regions, respectively, of the predicted or determined amino acid sequences of five other polypeptide chains: a chain encoded by pHDS4/203, the β -chain encoded by pHDS11 (ref. 13), 93G7 γ 1 immunoglobulin heavy chain, MOPC603 κ light chain, and MOPC104E λ 1 light chain²⁶. Those residues common between the pHDS58 protein and any one of the other five chains are boxed. *. The residues common among all six chains. Approximate boundaries of framework and hypervariable regions as they appear in immunoglobulin V regions are indicated by horizontal arrows. The amino acid positions refer to those of pHDS58 protein.

receptor β -chain deduced from pHDS11 and the polypeptide chain encoded by pHDS4/203, as well as to the sequences of an immunoglobulin heavy chain (93G7), an immunoglobulin κ light chain (MOPC603) and an immunoglobulin λ 1 light chain (MOPC104E)²⁶.

The homology is shown in Figs 5 and 6, where the pHDS58 amino acid sequence is compared with the V (or V + D), J and C region sequences of the other five polypeptide chains. Sequence homology is evident in all three regions, but is most striking in the J region, where it amounts to 38–62%. In the V regions, the pHDS58 sequence is related to the other five sequences by 22–29% homology. The six amino acid residues that are conserved in this region among pHDS11, pHDS4/203 and the three types of immunoglobulin chains (Gln, Cys, Trp, Gln, Tyr and Cys at positions 5, 22, 34, 37, 88 and 90, respectively) are also shared by pHDS58. The sequence homology is least in the C regions, where it ranges over 12–20%. Nevertheless, the relatedness is evident around the two Cys residues that form intradomain disulphide bonds in immunoglobulin chains. Throughout the V, J and C regions, the pHDS58 sequence, like the pHDS11 and pHDS4/203 sequences, is more homologous to the light chains (25% and 27% for κ and λ , respectively) than to the heavy chain (23%).

Beyond the C region, the pHDS58 polypeptide chain exhibits no obvious sequence homology to the corresponding regions of the β -chain (pHDS11) or the chain encoded by pHDS4/203. Nevertheless, the three polypeptide chains are organized in a very similar fashion in these regions. Thus, as in the pHDS11 and pHDS4/203 chains, the C region of the pHDS58 chain is followed by a peptide carrying an extra cysteine, then by a stretch of about 20 hydrophobic residues that could correspond to a transmembrane peptide and finally by a short hydrophilic C-terminal peptide that presumably extrudes into the intracytoplasmic space.

In Fig. 4 these regions of pHDS58 are indicated by horizontal arrows. The exact boundaries of a few adjacent regions are somewhat uncertain. For instance, it is not possible to determine unambiguously the N-terminus of the processed protein from the nucleotide sequence of the cDNA alone. However, the Gln at nucleotide positions 116–118 is the best candidate for the following reasons. First, the proposed assignment places the first Cys residue at position 22, while the corresponding Cys

residues of most immunoglobulin chains are at position 22 or 23 (ref. 26). Second, most immunoglobulin chains carry a signal peptide 19–22 residues long²⁶; the present assignment makes the proposed signal peptide 20 residues long. Finally, the pHDS58 chain is an excellent candidate for the α -subunit of the T-cell receptor, whose N-terminal residue has recently been shown to be blocked (S. Schlossman, personal communication), probably by a cyclized glutamine residue.

The proposed processed polypeptide chain is 248 amino acids long and its calculated relative molecular mass is ~28,000. The chain contains 23 negatively charged (15 Asp and 8 Glu) and 19 positively charged residues (6 Arg and 13 Lys), corresponding to an isoelectric point near neutrality in the absence of post-translation modifications. As shown in Fig. 4, it has four potential sites for N-glycosylation.

Conclusions

The pHDS58 gene and its product share several characteristics with the two previously identified T-cell genes, pHDS11 and pHDS4/203, and their products. (1) The gene is specifically expressed in various CTL clones but not in B lymphomas. (2) The gene is rearranged in CTL clones but not in myelomas and the rearrangement pattern varies with the CTL clone. (3) The rearranging poly(A)⁺ RNA is composed of 5'-variable and 3'-constant regions. (4) The primary sequence suggests that the encoded protein is composed of a signal peptide, two immunoglobulin-like domains, each with one disulphide loop,

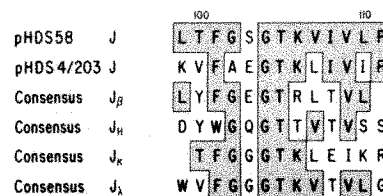


Fig. 6 Comparison of the J region amino acid sequences of pHDS58 and pHDS4/203 (ref. 13) and consensus sequences of the T-cell antigen receptor β -chain¹⁶ and immunoglobulin J_H , J_κ and J_λ (ref. 26). Those residues common between pHDS58 and any one of the other five J segments are boxed and shaded.

a transmembrane peptide and a cytoplasmic peptide. (5) The two domains are homologous to the V and C domains of immunoglobulin chains, particularly those of λ light chains. (6) Besides having two cysteine residues in each domain, the encoded protein has a fifth cysteine residue in the region between the C domain and the transmembrane peptide, that is, at a position where the chain might be disulphide bonded to another chain¹³.

The counterparts of the pHDS11 gene in a mouse T-helper hybridoma and a human T-cell tumour have been identified previously by Hedrick *et al.*¹² and Yanagi *et al.*¹¹ and shown to code for the β -subunit of the heterodimeric T-cell receptor¹⁴. As the nucleotide sequences of pHDS11 and the gene identified by Hedrick *et al.*¹² are virtually identical in the C region, we concluded that pHDS11 codes for the β -subunit of the CTL receptor¹³.

Because pHDS4/203, the second type of rearranged gene discovered in the CTL clone 2C, had many characteristics expected for a T-cell receptor gene, it was originally thought to code for the other (α) subunit of the CTL receptor¹³. Subsequently, endoglycosidase F digestion of the $\alpha\beta$ heterodimers of mouse helper T cells and a human T-cell tumour suggested that both subunits of these T-cell receptors are N-glycosylated (J. P. Allison, personal communication; C. A. Janeway, personal communication), but the pHDS4/203 gene, unlike the pHDS11 gene, has no typical N-glycosylation sites (namely, the tripeptide

Asn-X-Ser/Thr)¹⁵. Although the difference in the source of T cells (that is, CTL versus helper and mouse versus human) could have explained the apparent discrepancy, our recent studies suggest that the α -subunit of CTL clone 2C is also N-glycosylated (unpublished data). It is therefore of particular interest that the newly discovered pHDS58 gene reported here not only possesses all of the properties expected for a T-cell receptor gene but also carries four potential N-glycosylation sites. Thus, while there is no direct evidence, the pHDS58 gene must be considered a better candidate than pHDS4/203 for the α -subunit.

If the pHDS58 gene encodes the α -subunit and pHDS11 the β -subunit of 2C clone $\alpha\beta$ heterodimeric receptor, what is the function of the pHDS4/203 gene? As this gene exhibits clonal diversity, in common with the other two genes, it also is likely to be involved directly in determining T-cell specificity. If so, this raises the possibility of a second receptor. The idea of a second receptor has been previously discussed at length²⁷⁻³² but is at present less favoured than the one-receptor hypothesis³³⁻³⁶. The role of the third gene should be clarified by analysis of its product, currently underway.

We thank John McMaster and Mark Pichler for technical assistance, Eleanor Basel for secretarial assistance and Sifri Novotny for discussions. The work was supported by grants CA-28900-04 (S.T.), CA-28900 (H.N.E.) and CA-14051 (a core grant to S. E. Luria). D.M.K. is supported by the Arthritis Foundation.

Received 4 October; accepted 11 October 1984.

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Structure, expression and divergence of T-cell receptor β -chain variable regions

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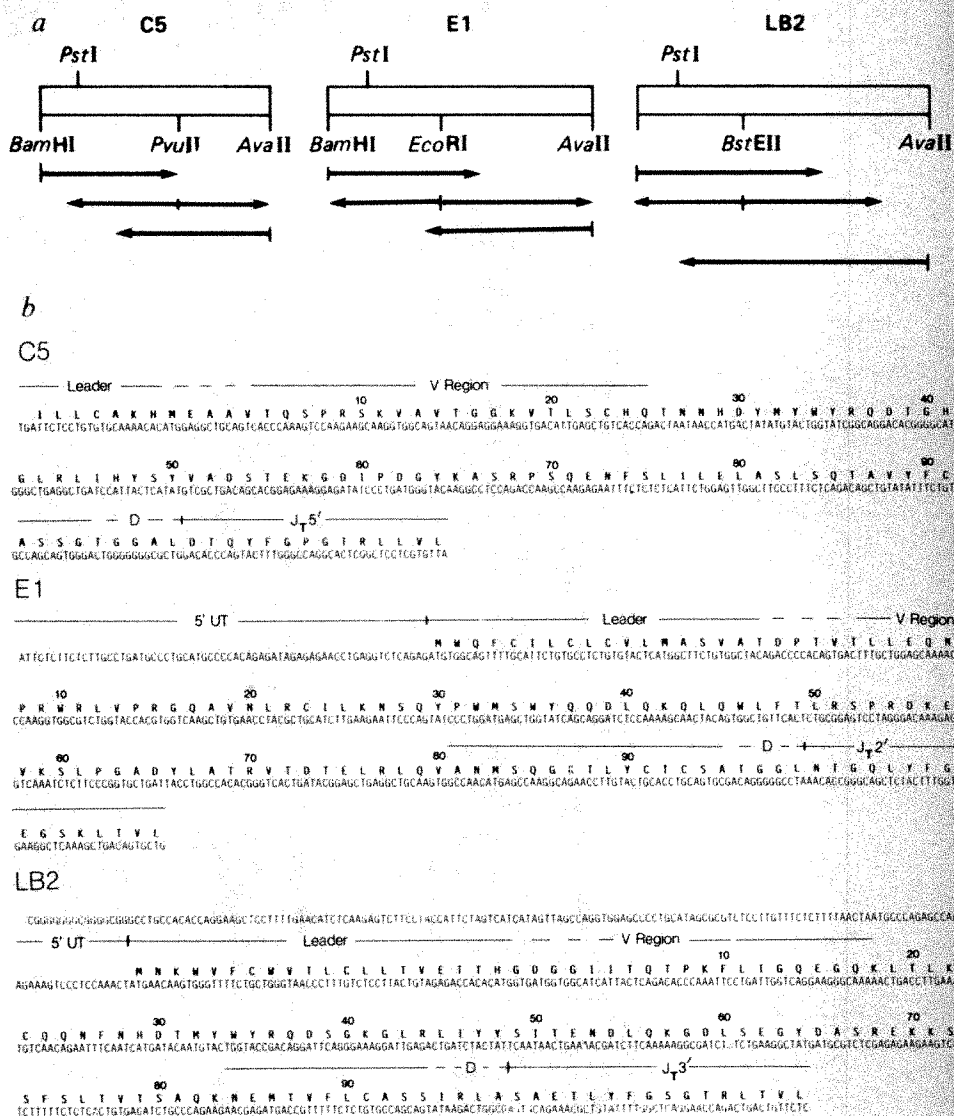
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Analysis of three new T-cell receptor β -chain variable regions together with those in the literature indicates that they have both remarkable similarities and differences with those of immunoglobulin. Less than 10 V regions appear to predominate in the thymus. V β sequences are much more heterogeneous at the amino acid level than are immunoglobulin V regions and they appear to diverge between species much more quickly, apparently the result of additional hypervariable regions. Three of these putative new hypervariable regions lie outside of the classical immunoglobulin binding site, an indication that important interactions may be occurring in these regions with polymorphic MHC determinants.

RECOGNITION of foreign entities by T cells appears to involve molecules and mechanisms strikingly similar to those involving immunoglobulins. Analysis of T-cell receptor β -chain cDNA clones and the genes encoding them has revealed V, D, J and

C regions similar in size and sequence to the corresponding immunoglobulin elements¹⁻¹⁰. The V, D and J genomic elements are rearranged during T-cell differentiation to form a single exon, as is the case for immunoglobulins and this rearrangement



appears to be mediated by flanking sequences analogous to those of immunoglobulins⁴⁻⁸. However, these studies have not revealed how the T-cell receptor polypeptides differ significantly from immunoglobulins to account for the recognition phenomena unique to T cells. Specifically, the structural bases of the requirement for simultaneous recognition of both antigen and self-major histocompatibility complex (MHC) determinants and the antigen-independent process of 'learning' self-MHC, which presumably occurs in the thymus^{11,12}, remain obscure. To address these questions, we studied the V-region sequences of β -chains isolated from various T-cell lines and hybridomas. We have begun to define the putative binding site(s) of this molecule by identifying framework (conserved) and hypervariable (nonconserved) regions, according to the precedent of Wu and Kabat¹³ in immunoglobulins. We also demonstrate that β -chain variable regions appear to diverge between species much more rapidly than immunoglobulin variable regions, consistent with a larger fraction of the molecules encoded by the former being devoted to interaction with variable determinants (MHC and antigen). In addition, we have used these V regions to assess the overall repertoire of T-cell specificities in the thymus through the frequency with which these V regions are used in thymic T-cell populations.

β -Chain cDNA clones from helper T-cell lines

T-cell lines generated by growing fetal liver and/or bone marrow cell suspensions in conditioned media have proved to be an important source of cloned, non-transformed cells of defined specificity. cDNA libraries from four such cell lines¹⁴⁻¹⁶ were

constructed (as described by Okayama and Berg¹⁷) from poly(A)⁺ RNA made from concanavalin A (Con A)-stimulated cells, using the pcD vector¹⁸. Three of the cell lines have a T-helper (T_h) phenotype in that they respond to antigen and class II MHC determinants by secreting growth factors¹⁵⁻¹⁶. These lines are E1, which is reactive to trinitrophenol (TNP) on a BALB/c MHC background (I-A^d-restricted)¹⁵; C5, which responds to the dinitrophenol (DNP)-ovalbumin conjugate on a BL/6 background (I-A^b-restricted)¹⁵; and LB2, which recognizes chicken red blood cells, also on a BL/6 background (I region-restricted)¹⁶. About 0.1-0.4% of the cDNA clones are positive for the β -chain T-cell receptor gene, a frequency similar to that of a total thymocyte cDNA library and considerably higher than that of T-helper cell hybridomas (0.01-0.02%)^{4,19}. This higher level of transcription of the β -chain in T helper lines is consistent with reports that cell lines, in contrast to hybridomas, have been shown to bind antigen and hence may have more T-cell receptor protein on their cell surface than hybridomas²⁰. A fourth cDNA library was constructed from the RNA of a suppressor T-cell line, Ly 23/4 (ref. 14) and had a similarly high level of β -chain-positive cDNA clones. Of 25 such cDNA clones surveyed, none appeared to have V regions. Instead, all 25 clones were in a size range expected for transcripts from D-J joining events^{7,9} and sequence data from one of these clones confirmed this interpretation (data not shown). This is in contrast to the half to two-thirds of the β -positive cDNA clones from the other libraries which have V regions, and may be related to the apparent absence of β -chain expression in murine T-suppressor hybridomas²¹.

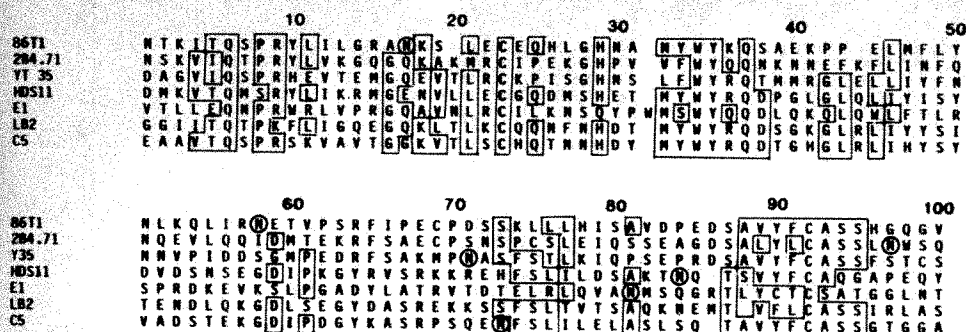


Fig. 2 Amino acid homologies between the seven β -chain variable-region sequences available. Homologies between four or more sequences are boxed. Both V- and D-region sequences are shown. Potential N-linked glycosylation sites are circled.

cDNA clones with complete V_β coding regions were isolated from the E1, C5 and LB2 libraries and their nucleotide sequences were determined by the method of Maxam and Gilbert²² according to the strategy in Fig. 1. All of these transcripts derive from the 3' most $J-C$ locus ($J_7\beta'-C_7\beta'$)⁶, as do the sequences of all four of the other full-length cDNAs derived from functional lines, hybrids or lymphomas^{4,23,24}. Although cDNA clones in the spleen or thymus appear to rearrange equally well to either of the two loci, at least in the context of a specific V region²⁴, few functional T-cell lines appear to use the 5' most locus (G. K. Sim, personal communication, N. R. J. Gascoigne and J. Elliot, personal communication). As the two C regions are almost identical⁶, the strong bias of such cells for the 3' most locus may reflect a selection for J regions from that cluster and may be an important clue in determining the functional significance of a tandem $J-C$ arrangement of the B -chain locus.

The data presented in Fig. 1 also show that E1 and C5, though reactive with structurally related antigens, share very little sequence homology. This lack of homology may reflect the effect of carrier, as C5 is conjugate-specific for DNP-ovalbumin whereas E1 is hapten-specific for TNP. Alternatively, it could reflect the effect of different restricting alleles (E1, I-A^d; C5, I-A^b) on antigen binding. Another explanation is that the other chain of the receptor (α) binds antigen in these clones. In addition to the lack of homology between the β -chain variable regions of E1 and C5, there is no homology in any portion of these with the antigen-binding portions of known TNP- or DNP-binding immunoglobulins²⁵—a further indication of the very different requirements of T-cell and B-cell recognition and an indication of why receptor isolation schemes involving T- and B-cell lines of matched antigen specificity were unsuccessful²⁶⁻²⁸.

β -Chain V regions in thymus

To assess the relative contributions of various β -chain V regions to the T-cell receptor repertoire, V region-specific fragments were prepared from each of the three cDNA clones described

above; from 2B4.71, a cytochrome c -specific T_H hybrid⁴; and from 86T1 (ref. 2), isolated from a thymocyte cDNA library (see legend to Table 1). These five distinct V-region fragments were labelled and used to screen 250,000 λ gt10 (ref. 29) cDNA clones from a BALB/c strain thymocyte poly(A)⁺ RNA-derived cDNA library (200,000 independent clones). Table 1 shows the percentage of clones in this library that were positive for both the β -chain constant region and these V regions. This analysis indicates that four of the five V regions fall into a uniformly frequent class of transcripts, each representing 1–3% of the C-region hybridizing clones. Since four of these five genes hybridize to single bands, and the fifth to only two bands in genomic Southern blots (see below), these frequencies must largely reflect the expression levels of the loci from which they were derived. The actual frequency of these V_β sequences is even greater since, of 35 C_β-positive clones analysed from this library, only six extended sufficiently far 5' to have hybridizable V regions (data not shown), presumably due to frequent D–J joining events^{7,9} and a 3'-end bias in cloning. The corrected frequencies are indicated in Table 1. Therefore, these four V regions together comprise 8.3% of the total β -chain message, and an even larger fraction (~40%) of functional transcripts. Thus, a relatively small number of V regions, perhaps less than ten, predominate in the thymus. The notion of a small V_β repertoire is supported by the fact that among the six murine V_β s reported here, two have been isolated independently from other functional T cells (86T1²⁴ and LB2, S. Hedrick, personal communication).

In contrast, the 2B4 V region was not detected among 250,000 clones surveyed, and thus must be expressed at a frequency at least 20 times lower. This is consistent with the observation that cytochrome c is a relatively weak antigen and reactive cells are only observed in secondary responses (R. Schwartz, personal communication). There are many such weakly reactive antigens^{30,31} and they may represent a second group of infrequently occurring V regions of unknown repertoire size. The regulatory mechanisms generating such uniformity in the frequency of the abundant class as opposed to the 2B4.71 V region are unknown. This selective expression contrasts with the much more diverse expression of immunoglobulin V regions in B cells, although a single heavy-chain variable region (V_H) seems to be expressed very frequently in early B cells (F. Alt, personal communication).

Additional hypervariable regions

With the sequences presented here, there are now seven β -chain V regions available^{2-4,23} for comparison with respect to framework and hypervariable regions. Homologies between the seven sequences are shown in Fig. 2 and the relevant numerical

Table 1 Variable region usage in a BALB/c thymocyte cDNA library

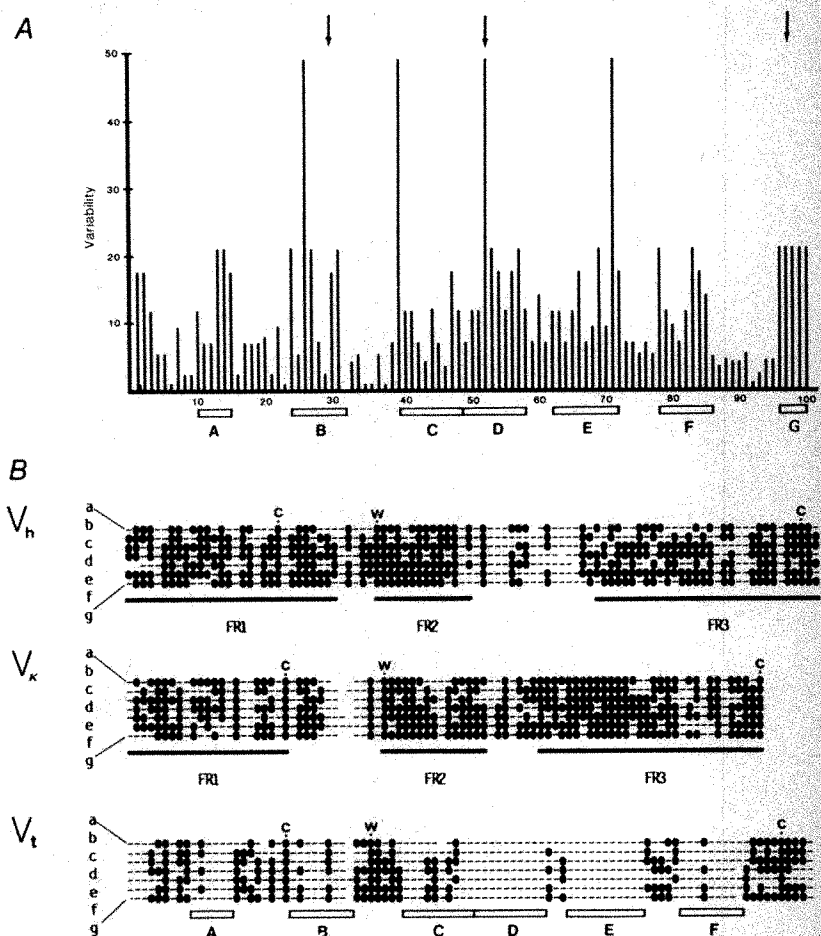
Variable region	Frequency (%)	Corrected frequency
C5	3.2	9.3%
E1	2.2	12.8%
LB2	0.9	5.2%
86T1	2.0	11.6%
2B4.71	<0.05	—
Total	8.3	38.9%

The following V region and C_β-specific fragments were purified on polyacrylamide gels and nick-translated by standard procedures⁴⁷: 86T1, *EcoRI*–*Bam*HI; 2B4.71, *EcoRI*–*Rsa*I; C5, *Pst*I–*Pvu*II; E1, *Pst*I–*Pst*I; LB2, *Bst*EII–*Xho*I; C_β 86T5², *EcoRI*–*Eco*RI. There were 1,650 C_β-positive clones. The percentage of these that were positive for a given V region is indicated. The corrected frequency is the raw percentage divided by the fraction of C_β-positive cDNA clones bearing V regions. The corrected value for C5 was divided by two as it hybridizes to two bands in genomic Southern blots and hence may cross-hybridize with another expressed V region.

Table 2 Variable-region homologies (%)

	2B4	YT35	HDS11	E1	LB2	C5
86T1	36	33	28	18	30	28
2B4		44	18	18	23	21
YT35			26	19	26	35
HDS11				18	44	51
E1					18	20
LB2						48

Fig. 3 Hypervariable and framework regions. **A**, Variability plot of the T-cell receptor β -chain V-region sequences shown in Fig. 2, using the convention of Wu and Kabat¹³. The positions of the three hypervariable peaks of immunoglobulin light chains²⁵ are indicated by arrows. Regions of hypervariability are marked by open bars. **B**, **D** and **G** correspond to the classical immunoglobulin hypervariable regions²⁵. Part **B** indicates the homologies between a variety of V_H , V_κ and the seven V_β sequences. Homologies of four or better are indicated by a closed circle, less than four homologies by a dash, and spaces denote gaps inserted to maximize homology. Framework regions of the immunoglobulin sequences are indicated with solid bars. **A**, **C**, **E** and **F** are regions of V_β sequence that correspond to conserved V_κ framework residues, but are significantly more variable in V_β . With the exception of the V_H region of *Caiman crocodylus*³³, sequences are from ref. 25. Immunoglobulin heavy chain (V_H): a, mouse group I MOPC 141CL; b, mouse group II MOPC 104E; c, mouse group III TEPC 601; d, dog MOO; e, rabbit K25; f, human VH26; g, caiman pC3. Immunoglobulin κ -chain (V_κ): a, mouse 10K26-12A1; b, mouse S107B CL; c, dog GOM; d, human group I ROY; e, human group III, 1T1; f, rabbit K9 335; g, rabbit K25. V_T : a, 86T1; b, 2B4.71; c, YT35; d, HDS11; e, E1; f, LB2; g, C5.



comparisons in Table 2. Whereas the least related immunoglobulin V regions are 45% homologous in amino acid sequence and range up to 98% (ref. 25), the most similar V_β regions shown here are 51% homologous and range as low as 18% (Table 2). Although it has been suggested²³ that some of this variability is due to helper and cytotoxic T cells using different sets of V regions, this comparison shows that equally low homologies are observed between T_H -derived V_β 's, and that the cytotoxic T cell-derived V_β HDS11 has 51% amino acid homology with the T_H -derived V_β C5—the highest homology observed between any two V_β s in Table 2. Furthermore, the helper-derived V_β LB2 is nearly identical to nucleotide sequence (one change in 110 nucleotides examined) to a V_β derived from a functional cytotoxic T cell (S. Hedrick, personal communication).

These relatively low levels of homology among β -chain V regions are further analysed in the Wu-Kabat variability plot in Fig. 3A; this follows the formula (no. of different amino acids)/(frequency of the most common amino acid)¹³ and serves to highlight regions of maximum variability. Both heavy- and light-chain immunoglobulins exhibit three hypervariable peaks when plotted in this manner, two in the main body of the V region (CDR1 and CDR2) and a third corresponding to the V-J joint in light chains and the V-D-J joint in heavy chains²⁵ (indicated by arrows in Fig. 3A). In contrast, the V_β sequences examined in Fig. 3 exhibit seven distinct variability peaks, three of which correspond almost exactly to the canonical immunoglobulin κ light-chain hypervariable regions. Two of the peaks (A and F) correspond to regions in V_H and V_κ that have been shown to be significantly more variable than classical framework regions^{25,32} but less variable than the classical hypervariable regions. The third and fifth peaks (C, E), however, do not have any precedent in immunoglobulins.

Complementary to these variability data are the homology comparisons shown in Fig. 3B; in this representation, four or more identical residues out of seven are displayed as solid circles

and fewer than four as dashes. In a control for the validity of conclusions based on a small number of sequences, a similar comparison is made of seven V_κ and V_H sequences^{25,33}, chosen for maximum variability. In this analysis, the classical hypervariable (HV) and framework (FR) regions of immunoglobulins (indicated by solid bars under immunoglobulin sequences) are picked up remarkably well. By comparison, the β -chain V regions have stretches of homology flanking the conserved tryptophan and cysteines that are comparable with those in immunoglobulin FR regions. However, there are four new regions of sequence, corresponding to the four new peaks of variability in Fig. 3A, whose V_κ equivalents are conserved FR residues, but which are much more variable in V_β (Fig. 3B, regions A, C, E, F). As discussed below, the sequence variability in these regions may have important consequences for antigen-MHC interactions.

Rapid divergence of β -chain V regions

There are two interpretations that are consistent with the remarkably low levels of homology between the V_β s reported here relative to immunoglobulin V regions. Cellular immunity could significantly predate humoral immunity and V_β s could evolve at a rate comparable to immunoglobulin V regions; the difference in homology between V_β s being then a result of having diverged from each other much earlier than immunoglobulin V regions. Alternatively, there could be significantly different selective pressures on V_β s as a consequence of the requirements of Ag-MHC interactions unique to the T-cell receptor. In the case of immunoglobulin, those portions of the V_H regions that interact with antigen (HV regions) acquire mutations at a much greater rate than do framework regions. Specifically, HV regions appear to diverge at the rate of unselected DNA, whereas FR regions evolve at a rate comparable to globin genes³⁴. These HV regions make up 22% of both V_H and V_κ ²⁵, whereas the analysis in Fig. 3 shows that ~54% of V_β may be hypervariable. If these putative new HV regions evolve at a rate comparable to those

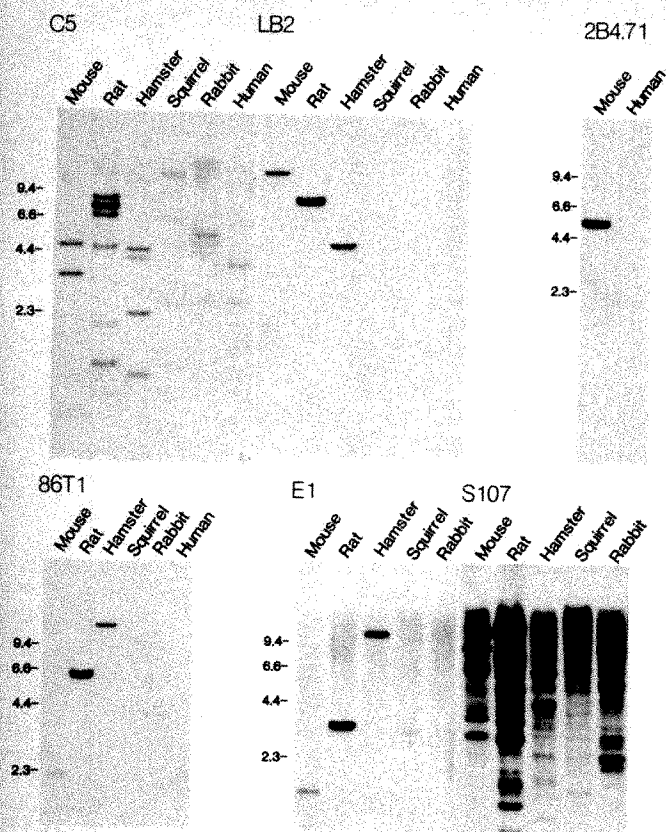


Fig. 4 Interspecies homologies of β -chain variable regions. *Eco*RI digests of mouse (BALB/c), rat, hamster (CHO), ground squirrel, rabbit and human DNAs were electrophoresed through 0.9% agarose and blotted onto nitrocellulose by standard procedures⁴⁷. V-region specific fragments from five of the β -chain clones and one from an immunoglobulin heavy chain (S107 V), were nick-translated and hybridized overnight at 55 °C in $6 \times$ SSC and washed at the same temperature, the final wash using $0.2 \times$ SSC for 30 min. Size markers are indicated in kilobases. Divergence time from mice (millions of years)^{36,37}: rat, 15 Myr; hamster, 25 Myr; ground squirrel, 60 Myr; rabbit, 70 Myr; human, 90 Myr.

of immunoglobulin, the V_{β} gene as a whole should evolve much more rapidly than V_H or V_{κ} genes.

To resolve between these interpretations, we used nucleic acid hybridization as an assay for the divergence of five murine V_{β} s and a representative V_H (S107)³⁵ from their homologues in other species. We reasoned that if a much larger fraction of V_{β} s is indeed hypervariable, hybridization of V region-specific probes with their homologues in successively more distantly related species should fall off much more rapidly for V_{β} than for V_H . Figure 4 shows a survey of six mammalian genomes, spanning 90-Myr of evolutionary time, probed on Southern blots with purified V_{β} probes and the S107 V region. Evidence that the rate of divergence of V_{β} s is in fact greater than that of V_H s is indicated by the fact that in general the V_{β} probes only hybridize well with closely related rodent genomes^{36,37}. S107 V_H , on the other hand, hybridizes well with squirrel (60 Myr divergence)³⁷ and rabbit (70 Myr divergence)³⁶ on comparable blots. Furthermore, S107 V_H and an independent V_H probe, μ 107 V (discussed in ref. 38), have been shown to hybridize well in more stringent conditions to much more distantly related genomes³⁸, including the primitive reptile, *Caiman crocodylus* (300 Myr divergence from mouse³⁶). Murine V_{κ} probes have also been shown to cross-hybridize well on human genomic Southern blots³⁹. Taken together, these data indicate that most V_{β} s acquire mutations at a significantly greater rate than V_H s and V_{κ} s¹⁵. While Southern blots do not provide the resolution of sequence data, this observation is consistent with the notion that a greater portion of V_{β} s are devoted to interaction with variable determinants and are thus selected to be hypervariable.

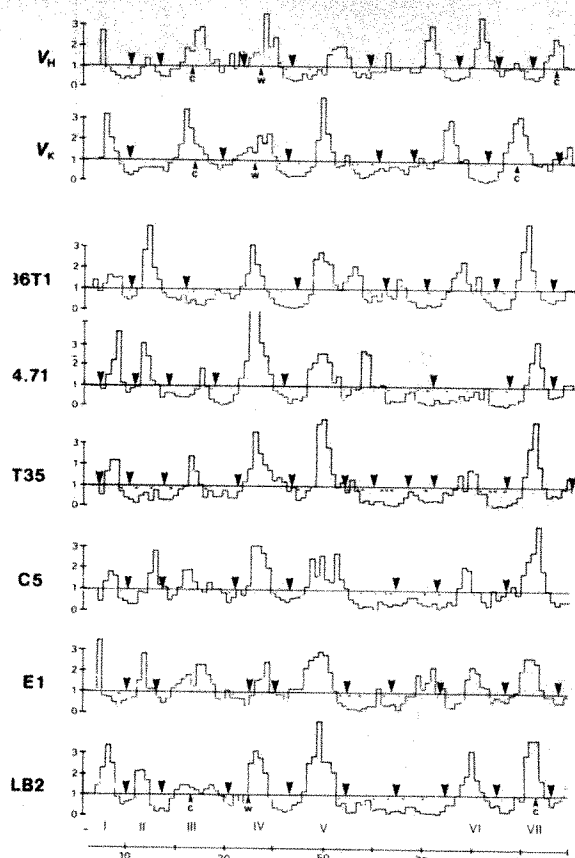


Fig. 5 Secondary structure predictions. The algorithm of Chou and Fasman⁴² was used to predict the locations of probable β -sheets and turns in the V_{β} sequences and in representative immunoglobulin sequences²⁵. P_{β} , plotted on the vertical axis, represents the β -sheet conformational parameter as defined⁴². Arrows are centred on regions of sequence where $P_T > P_{\beta}$ and $p_T > 0.75 \times 10^{-4}$. In regions where turns were predicted to be directly adjacent or to share amino acids, only the most probable turn is indicated by an arrow.

Consistent with an increased rate of divergence is the fact that four of the five V_{β} regions hybridize to single bands in the genome compared with between 4 and 30 bands that are observed with V_H ⁴⁰ and V_{κ} ^{39,41} probes. That we see single hybridizing bands does not rule out the possibility that there are families of related V_{β} sequences; it suggests, rather, that members of a given family have probably diverged too much from each other to cross-hybridize at the relatively moderate stringencies used here. The exception to this rule, C5, lends support to the above interpretation. It is the only V region that is conserved enough between species to hybridize well to squirrel, rabbit and human genomic DNAs, consistent with the observation that it is also the only V region to hybridize to multiple bands in the species surveyed³⁻⁸. The simplest interpretation of these hybridization patterns is that variation in some V_{β} sequences is under more stringent constraints than others, and that such V_{β} regions will therefore have retained enough sequence homology between duplicated copies in a given genome to appear as gene families. It also suggests that very low-stringency hybridization experiments may reveal that some of the other V_{β} sequences are also members of homologous gene families.

Homology with immunoglobulin

Based on amino acid homology, the published sequence of 86T1 more closely resembles a variable region from a κ chain (McPC 603, 33 of 84 amino acids homologous) than a variable domain from either a λ (MOPC 104e, 25 of 86) or heavy chain (93G7, 26 of 90)². Each of the additional V_{β} s reported here is more

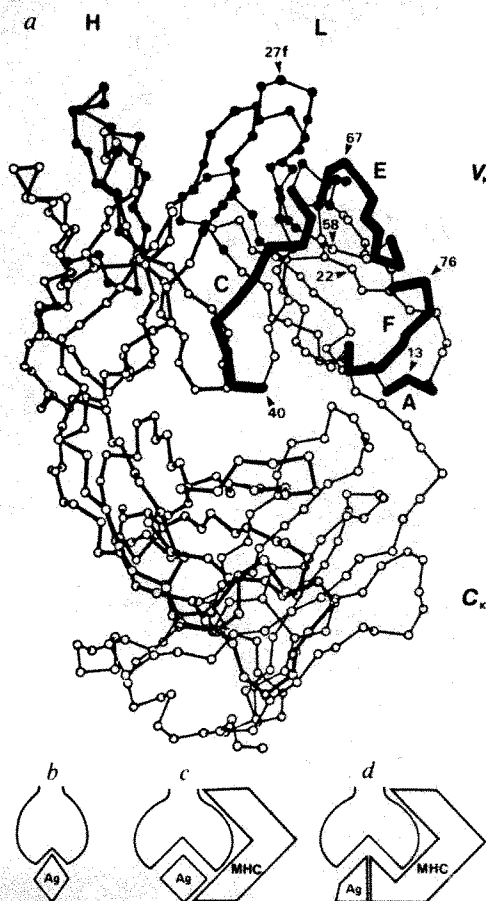


Fig. 6 Possible T-cell receptor structure and implications for recognition. *a*, An α -carbon skeleton of the X-ray crystallographic structure of McPC 603 (ref. 43). Thick lines denote heavy-chain residues. Closed circles correspond to classical hypervariable residues. The regions of sequence identified in Fig. 3*B* (A, C, E, F) that correspond to V_k framework regions, but are significantly more variable in V_β s, are shown as solid bars. *b*, A schematic diagram of antibody-antigen (Ag) interaction; *c*, *d*, models of T-cell receptor binding.

homologous with this κ V region than with these λ or heavy chains. Among the conserved residues that form the interface between V_H and V_L ⁴³, the T-cell receptor conserves the same amino acids at more of the light-chain contact residues (Tyr 36, Gln 38, Leu 46) than those of heavy chain (Gln 39, Leu 45). By these criteria, V_β most resembles a κ variable region. The only structural features which are more heavy chain-like are the number of amino acids from the invariant tryptophan to the last cysteine of the V region (4 or 5 residues shorter in V_κ than in V_β) and the fact that the last V_β framework region extends several amino acids past this cysteine, as in V_H . These similarities of V_β to V_H at the carboxy-terminal end may relate to the presence of D region elements^{4,5} and to the larger J regions characteristic of heavy chains^{4,6}. The intron-exon structure of the constant region with its separate hinge and transmembrane regions is also more heavy chain-like⁶.

Despite the similarities in amino acid sequence between V_β and V_κ , the relative lack of primary structure conservation among V_β s makes it important to determine whether the predicted secondary structure of these V_β s differs significantly from the known structure of V_κ and whether secondary structure is predicted to be conserved among V_β s. We therefore used the empirically derived algorithm of Chou and Fasman⁴² to locate regions of sequence predicted to be probable β -sheets or β -turns. We assumed that V_β s, like V_H and V_κ , have little or no α -helical content. Figure 5 shows the β -sheets and turns predicted by the algorithm of Chou and Fasman for six of the seven V_β s reported here. The predicted secondary structures of representative heavy- and light-chain V regions are included for comparison.

This analysis reveals that, among these V_β s, seven β -sheets are consistently predicted (five of six or better) in regions of sequence that correspond to β -sheets in immunoglobulin. The only apparent departure of the V_β predicted secondary structure from the known immunoglobulin structure is in residues 65–69 whose V_κ counterparts are known to comprise a β -sheet, but are predicted to be a sheet in only one of six V_β s (E1). The lack of a β -sheet in this region of sequence has precedent in immunoglobulin, as the Chou–Fasman algorithm often fails to predict the corresponding sheet in κ (unpublished observation). Thus, despite the extreme variability in primary structure between V_β s, the secondary structure is predicted to be conserved and to be immunoglobulin-like.

Models of T-cell receptor binding

Because of the similarities in the primary and predicted secondary structure between the T-cell receptor and the extensively characterized immunoglobulins, it is instructive to ask where, by analogy with immunoglobulin, putative new V_β hypervariable regions lie on the molecule. Historically, the assumption that regions of sequence which are highly variable among many immunoglobulins correspond to portions of the molecule which make physical contact with antigen has been a useful principle in analysing the antigen-binding site of immunoglobulins¹³. We have therefore applied this heuristic to the T-cell receptor β -chain in order to derive the implications for antigen–MHC interactions from the predicted location of putative new HV regions. Adapted from ref. 43 is an α -carbon skeleton of an immunoglobulin Fab from the myeloma McPC 603. Classical hypervariable regions are indicated on this Fab model by closed circles; regions of V_κ that correspond to putative new V_β hypervariable regions are indicated by heavy bars. Regions A and F correspond to external loops between antiparallel β -sheets, as do classical immunoglobulin HV regions. Region E corresponds to a β -turn and sheet that interacts with solvent in the V_κ molecule. Curiously, these regions lie external to the classical immunoglobulin antigen-binding site. Their variability in V_β suggests that important interactions may be occurring on the outer surface of the receptor, perhaps with polymorphic histocompatibility determinants. Region C corresponds to a β -turn and sheet that is highly conserved at the amino acid level in V_κ and makes multiple hydrophobic interactions and hydrogen bonds with the immunoglobulin heavy chain⁴³. In the T-cell receptor, despite the greater variation in amino acid residues in this region, the secondary structure is predicted to be highly conserved (Fig. 5). Although based on a small number of sequences, the variability of these residues in V_β suggests that they interact with solvent, antigen or the MHC; alternatively, the other chain of the T-cell receptor (α) has complementary variability in the residues in contact with this region of V_β .

Present cellular evidence indicates that a single T-cell receptor is responsible for recognizing both MHC and antigenic determinants^{44–46}. Assuming that the binding site(s) of this receptor consists of the α - and β -chains and that they fold in an approximately immunoglobulin-like fashion, the localization of the putative new hypervariable regions (Fig. 6*a*) suggests that the T-cell receptor may have the properties shown schematically in Fig. 6*c*, *d*. Specifically, it may have a larger binding pocket than that of immunoglobulin and its outer surface may be in contact with polymorphic MHC determinants. An alternative model (not shown) proposes the interaction of MHC with the classical immunoglobulin-like binding site, and antigen interacting with the putative new HV regions on the outer surface of the receptor. These models predict that different MHC restriction elements could alter the antigen-binding properties of the β -chain through allosteric effects. Preliminary evidence with anti-cytochrome *c*-reactive hybridomas is consistent with these models, as different β -chain V regions are used against the same antigenic peptide in the context of different MHC restricting alleles (S. Hedrick, unpublished observation).

We thank Dr John Bell for his generous gift of human DNA, Drs Robert Schimke and Chuck Gasser for CHO DNA, Dr C.

Benoist for thymocyte cDNA libraries, Dr Tim Mosmann for LB2 cells and Dave Austin for the use of his immunoglobulin model. We also thank Dr Gordon Freeman for preparing E1 and C5 mRNA and for assistance in the cDNA cloning, Dr Steve Crews for S107 DNA and critical reading of the manuscript, Dr Elvin Kabat for critical remarks on the manuscript,

Received 31 August; accepted 20 September 1984.

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Dr Harvey Cantor for helpful discussions, and Kathy Redman and Gloria Samuel for help in preparing the manuscript. P.A.P. is supported by a NIH predoctoral training grant in molecular and cellular biology. Much of the work described here was funded by grants to M.M.D. from the NIH and the American Cancer Society.

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LETTERS TO NATURE

Detection of possible optical flashes from the γ -ray burst source GBS0526-66

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The unidentified source of the γ -ray burst of 5 March 1979, GBS0526-66, may be located in the supernova remnant N49 in the Large Magellanic Cloud¹, though some authors^{2,3} have questioned the implied distance of 55 kpc. Among several unusual features of the source (reviewed by Cline⁴) is its repeatability: a total of 16 γ -ray bursts have been reported³, which may be periodic⁵. Only γ -ray bursts have been detected from this source. The situation for other γ -ray burst sources is not very different. Optical transients are known, in three cases^{6,7}, to have occurred near positions of later γ -ray bursts. Even so, no source has been identified optically⁸⁻¹⁰, and only upper limits are known for simultaneous emission of optical radiation with γ -ray bursts¹¹. Here we report that GBS0526-66 was monitored for ~910 h, using a high speed photoelectric photometer, attached to a 50 cm telescope at ESO/La Silla. Three short optical flashes were found, which may possibly be related to the γ -ray burst phenomenon. The most intense of these occurred on 1984 February 8 UT 07:42. It reached a maximum brightness corresponding to $m(\text{visual}) = 8.7$. The shape of the light curve bears remarkable similarity to the initial 200 ms of the 1979 March γ -ray burst.

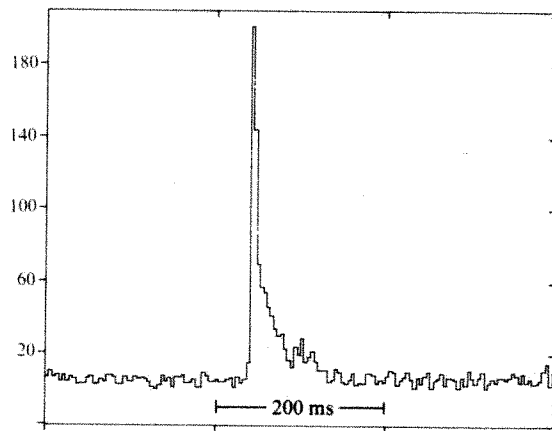


Fig. 1 An event observed 1983 October 27 UT 01:28. The time bin is 4 ms. Vertical axis corresponds to no. of counts, uncorrected for dark count or sky.

Photometric observations of the 1979 March γ -ray burst error box were made during the period 1983 October 5 to 1984 February 23. A single channel photometer was used with a rectangular entrance diaphragm, 20 by 80 arc s, aligned with the error box. Offset-guiding allowed the diaphragm to remain centred on the error box during almost all the observations and, in particular, during the burst moments discussed here. Most measurements were done through a Johnson V filter ($\lambda = 550$ nm, $\Delta\lambda = 80$ nm). During the period up to 1983 October 20, an EMI 6256 photomultiplier was used; later data were acquired with an RCA C31034A tube. Pulse counts were written on magnetic tape at equal time intervals. The time bin was 4 ms until 1983 November 26, and 1 ms afterwards. Integrations with a time-base of 4 ms were kept in floating-point words; those of 1 ms were packed with two integers per 16-bit word. The resulting maximum count rate $255,000 \text{ s}^{-1}$ corresponds to $m(\text{vis}) = 6.6$. A strip-chart recorder allowed immediate inspection of the count rate. The observer could set the time resolution, usually 1 or 0.5 s. During part of the observations, an on-line burst detecting procedure informed the observer about possible bursts in order that the sky conditions could be checked. An atomic beam standard frequency clock was used for time keeping. Absolute

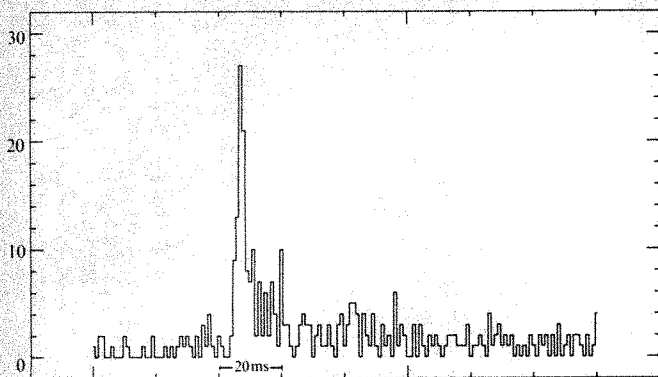


Fig. 2 An event observed on 1984 January 30 UT 03:20. The time bin is 1 ms.

time is presently known only to ± 100 ms because of an error in the calibration procedure. Data from 1983 November 2 to 19 were not recorded digitally, but only using the strip-chart recorder. The constancy of the system sensitivity was checked against the nearby star SAD249271, whereas photometric calibration was done using data from various E-region stars taken from ref. 12. The observations were carried out throughout the moon cycle, in spite of occasional light cloud cover.

A number of natural and man-made phenomena can cause spurious burst detection. Some, including electronic switching noise, lightning, the xenon flashlamps on aircraft, and meteors passing through the field of view, are of only millisecond duration, or less. We believe we have eliminated such effects by disregarding events lasting < 20 ms. Longer events, which may possibly mimic cosmic bursts, include the decay of luminescent meteor trains in the field of view, diffuse sky background illumination due to flaring fireballs, and the passage of artificial Earth satellites across the field of view. Little appears to be known about the first of these effects. Data published by Sekanina¹³, indicate that fireball light curves often have multiple maxima, and decrease in luminosity more rapidly than they increase, which is inconsistent with the events observed here. Slow-moving, artificial satellites would generally produce symmetrical light curves. Occasionally, a satellite may change brightness while moving only a few arcseconds, with unpredictable photometric results. Symmetric events of 32–90 ms duration have been observed on ten occasions, but we have not been able to demonstrate coincidence with a satellite passage. Finally we mention that Moon-illuminated clouds and contrails from jet aircraft can produce 'bursts' of a wide range of shapes and different durations. Contrails are particularly bothersome, as they may occur under all photometric conditions.

All data acquired during the 910 h observation period have been examined for the occurrence of highly significant events. The following three events comprise all flashes not suspected of being man-made, and of > 20 ms duration. Events discarded include the ten possible satellite passages, and a few chaotic events possibly attributable to low line-voltage. All three events were observed under perfect conditions—dark and clear sky; diaphragm well centred on error box. Their light curves are shown in Figs 1–3.

(1) 1983 October 27 UT 01:28 (Fig. 1): The burst was registered by the triggering routine. The two observers in the dome noted normal sky conditions. The rise time, half-width (FWHM) and total duration were < 4 , 8, and 90 ms, respectively. Maximum brightness was $m(\text{vis}) = 8.4$. The amount of energy in the first 4 ms time bin, relative to that in the tail, was $\sim 1/3$. This is possibly inconsistent with the detection of a meteorite passage, followed by the decay of its train. However, this explanation cannot be precluded. It is also possible that the rise time is in fact resolved: the bin preceding the maximum may indicate the start of the phenomenon. Alternatively it could be a statistical fluctuation.

(2) 1984 January 30 UT 03:20 (Fig. 2): The telescope was unattended. The rise time, half-width and total duration were

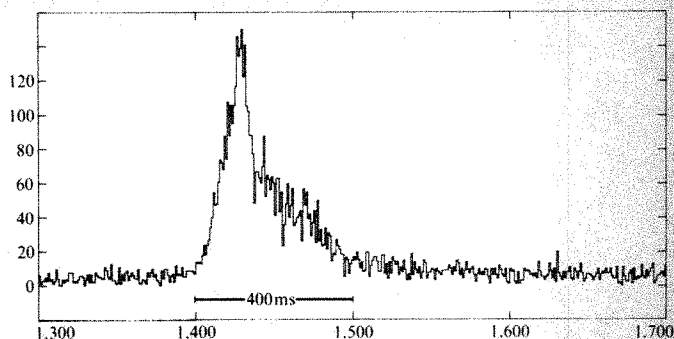


Fig. 3 An event observed on 1984 February 8 UT 07:42, at phase zero of the possible 164-day period determined by Rothschild and Lingenfelter⁵. The time bin is 4 ms.

3, 3, and 60 ms, respectively. The maximum brightness was $m(\text{vis}) = 9.9$.

(3) 1984 February 8 UT 07:42 (Fig. 3): The telescope was unattended. Maximum brightness was $m(\text{vis}) = 8.7$. The rise time, half-width and total duration were 110, 90 and 1,700 ms respectively. The event differs from the others by its much longer duration. Its resolved rising edge is a strong argument against a meteor explanation. The maximum brightness, integrated over the visible band, and referred to the top of the atmosphere, is $1.0 \times 10^{-9} \text{ erg s}^{-1} \text{ cm}^{-2}$. The corresponding fluence is $1.6 \times 10^{-10} \text{ erg cm}^{-2}$.

The simultaneous detection of γ -ray bursts and optical flashes would convincingly establish their cosmic origin. During our observations, four spacecraft with γ -ray detectors were in operation: ICE, PVO, SMM and Prognos 9. Preliminary analysis of these has revealed no response at the time of any of the optical flashes. The detector thresholds depend strongly on the time history and energy spectrum of a burst, but can be conservatively characterized by a fluence of $10^{-6} \text{ erg cm}^{-2}$, at energies above 100 keV. A pending analysis of real-time data may reduce this value by a factor of two. The absence of γ -ray burst detections is not surprising, given the thresholds and sensitivities of the satellites: none detected any of the soft, repeating bursts observed by the KONUS instruments. The inferred fluence ratio $E(\text{opt})/E(\gamma) > 1.6 \times 10^{-4}$ is consistent with similar, published data: Grindlay *et al.*¹¹ found the upper limit 10^{-3} for one particular γ -ray burst, which could not be detected optically, and Schaefer^{6,7} deduced values near 10^{-3} for the three historic, optical transients mentioned above.

In spite of the absence of γ -ray detections, several facts make the optical events remarkable: (i) Two of them have fast rise times and relatively slow decay, reminiscent of the 1979 March 5 event and of some of the recurrent bursts. (ii) The shape of the third burst is similar to the initial, impulsive phase of the 1979 March 5 γ -ray burst¹⁴. Both phenomena show a plateau at about half intensity, following the peak. The main difference is the finite rise time of the optical flash and its longer duration. (iii) The moment the third event occurred is consistent with the day of phase zero, as extrapolated from the formula given by Rothschild and Lingenfelter⁵: at this phase, a rather high burst rate may be expected.

We note that all published light curves of γ -ray bursts from GBS0526–66 (refs 1, 15–17) show very short rise times. If the 1984 February 8 optical flash represents reprocessed γ -rays from such rapid events, the rise time and half-width of the optical flash may be used to estimate a typical dimension of 30,000 km for the emitter. Unless the γ -ray bursts are strongly beamed, this seems to rule out reprocessing in the atmosphere of a (non-degenerate) neighbour star as proposed in the model of London and Cominsky¹⁸. However, it is consistent with small-disk models and with models involving cyclotron reprocessing. The latter have been considered by Woosley¹⁹.

We thank R. Lindskov-Simonsen and R. Castillo for help in making observations and data analysis; S. Laursen for work on hardware and software; the Danish Board for Astronomical

Research for a travel grant; and G. Andreoni for work with telescope tracking models.

Received 23 August; accepted 5 October 1984

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Is Geminga a very close neutron star binary?

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Geminga (2CG195+4) is one of the brightest sources in the sky at photon energies around 1 GeV (refs 1-3). It has been tentatively identified⁴ with an unusual soft X-ray and optical object for which the γ -ray-to-X-ray-to-optical luminosity ratios are $\sim 10^6:10^3:1$, which may now have been confirmed by the discovery⁵ of a ~ 60 -s periodic variation in the X-ray source, similar to that reported in γ rays. The period has been increasing over the past 10 yr at a rate such that it doubles in ~ 400 -800 yr. The implied rotational energy loss would supply the γ -ray luminosity of Geminga if the spinning object were a neutron star at a distance of ~ 10 pc, consistent with the very low observed X-ray absorption. A lone pulsar with a spin period of ~ 60 s cannot spin down at the required rate. The observed period would need to be due to precession or some other process. We show here that the presence of a close neutron star companion provides a suitable mechanism. All neutron star binaries may evolve through a Geminga-like phase.

A non-corotating conductor orbiting in a pulsar magnetosphere creates a circuit in which currents may flow along the field lines and through the neutron star (compare with the unipolar inductor of ref. 6). The currents will be driven by, and will tap energy from, the rotation of the pulsar. The optical and X-ray luminosities of Geminga are so small that plausible candidates for a conductor are severely restricted. A normal stellar companion would have to orbit at too great a distance and an ionized accretion disk (compare with ref. 7) or a white dwarf would be too luminous. We consider here that the conductor is another neutron star with a weaker, opposed, magnetic field. The 60-s period is identified with the difference between the spin frequency, F , of the neutron star (primary; with the larger magnetic moment, $\mathcal{M}_1$) and the orbital frequency of the other neutron star (secondary of magnetic moment $\mathcal{M}_2$). This orbital period is several minutes.

The common flux between two opposed coplanar dipoles of separation a is

$$\Phi = \frac{\mathcal{M}_1}{a} \alpha (1 - \alpha)^2 \int_0^{2\pi} \frac{\cos \phi \, d\phi}{(1 + \alpha^2 - 2\alpha \cos \phi)^{3/2}} \\ = 3\pi\alpha^2 \frac{\mathcal{M}_1}{a} f(\alpha) \quad (1)$$

where $\mathcal{M}_2 = \alpha^3 \mathcal{M}_1$, $f(\alpha)$ varies smoothly from 0.85 to 1 as α varies from 1 to 0. Hereafter we adopt $f(\alpha) = 1$. The effective cross-section of the secondary ($3\pi\alpha^2 a^2$ for small α) may greatly

exceed its geometric cross-section. The structure of the magnetic field is outlined in Fig. 1. The maximum voltage around the generator circuit occurs between the pair of 'last common field lines' coplanar with the two dipoles and the rotation axis. By mapping the field lines onto the primary, we obtain for small α

$$V_{\max} = 2\sqrt{3} \alpha \mathcal{M}_1 (F - \sqrt{GM/a^3})/a \\ \approx 3.5 \times 10^{12} \alpha \mathcal{M}_{21} a_8^{-1} \text{ V} \quad (2)$$

where $M = (M_1 + M_2)$ is the mass of the system, $a = 10^8 a_8$ m and $\mathcal{M}_1 = 10^{21} \mathcal{M}_{21} \text{ T m}^3$. A magnetic moment $> 2.5 \times 10^{21} \text{ T m}^3$ takes the surface field beyond the quantum limit where spontaneous pair creation is expected. A further small voltage difference of $\frac{3}{2} \alpha^2 \mathcal{M}_1 \omega'/a$ can occur if the spin of the secondary deviates from corotation by ω' .

The current flow in the magnetosphere cannot produce magnetic fields in excess of the dipolar ones^{8,9}, which in the present case limits the total current to less than

$$I_{\max} \approx 2\pi\alpha \mathcal{M}_1 / \mu_0 a^2 \\ = 5.0 \times 10^{11} \alpha \mathcal{M}_{21} a_8^{-2} \text{ A} \quad (3)$$

Deep within the crust of a neutron star, the electrical conductivity is isotropic and extremely large, but it is anisotropic at the surface if the magnetic moment is large^{10,11}. This region dominates the effective stellar resistance. A crude upper limit is obtained by applying a value for the surface conductivity ($\sigma_1 \approx 10^9 \text{ S m}^{-1}$, ref. 11) to the whole star;

$$R_{\text{eff}} < \frac{4a}{3\pi\alpha^2 r_*^2 \sigma_1} \\ \approx 4 \times 10^{-10} \alpha^{-2} a_8 \Omega \quad (4)$$

where r_* is 10 km. Since

$$R_{\text{eff}} \ll R_{\min} \approx V_{\max} / I_{\max} \\ = \frac{\sqrt{3}}{\pi} \frac{\omega a}{c} Z_0 \quad (5)$$

where $\omega = F - \sqrt{GM/a^3}$ and $Z_0 = \sqrt{\mu_0/\epsilon_0}$ ($\approx 38 \Omega$) the current is self-limited in the magnetosphere.

The power dissipated in the magnetosphere

$$P_m \approx V_{\max} I_{\max} = 4\sqrt{3} \pi \alpha^2 \mathcal{M}_1^2 \omega a^{-3} \mu_0^{-1} \\ = 1.7 \times 10^{24} \alpha^2 \mathcal{M}_{21}^2 \omega_{-1} a_8^{-3} \text{ W} \quad (6)$$

where $\omega = 0.1 \omega_{-1}$. It can be heuristically derived as the rate of working of the magnetic stress in the common flux tube. P_m should equal the power associated with the spin-down of the primary neutron star

$$P_R \approx 3.2 \times 10^{24} \text{ W} \quad (7)$$

for a period derivative of $3 \times 10^{-9} \text{ s s}^{-1}$, mass of $1 M_\odot$ and moment of inertia of $6 \times 10^{37} \text{ kg m}^2$. We propose that the electric potential ($\sim V_{\max}$) accelerates particles that radiate the observed γ rays. Synchrotron and curvature radiation from 1 to 10 TeV electrons can produce γ radiation in the appropriate range. Whatever the emission mechanism, its efficiency for γ -ray production must be very high ($> 99\%$), comparable with that of the pulsed emission from the Vela pulsar¹². We note that the large opacity to pair production on the magnetic field prevents γ rays emitted near the neutron star poles from escaping¹³. Ignoring beaming, the observed flux¹⁻³ of $2.4 \times 10^{-12} \text{ W m}^{-2}$ requires Geminga to be at a distance of ~ 11 pc.

A completely charge-separated beam will limit the current to $< I_{\max}$, since the potential variation across a current beam made of electrons or ions alone is roughly $\delta V \approx I/\epsilon_0 v_D > IZ_0$, where the drift velocity $v_D < c$. To attain P_m , we require the maximum current to flow and thus nearly neutral beams. This is achieved if the incoming particles dislodge charges from the neutron star surfaces. Pair production by γ rays in the intense magnetic field near to the neutron star may also supply a substantial fraction of the current carriers¹³.

The flux tube is an ellipse at the primary of semi-axes of $\sqrt{3/2}\alpha\sqrt{r_*^3/a}$ and $\sqrt{3}\alpha\sqrt{r_*^3/a}$, and for the parameters that give P_R its area, $A_\Phi \approx 4.7 \times 10^4 \alpha^m a_8^{-1} \text{ m}^2$, where $m = +2$ and -1 on the primary and secondary respectively. If the X-ray flux is due to black-body emission, we derive an area of $\approx 5 \times 10^4 T_6^{-2} \text{ m}^2$, where $T = 10^6 T_6 \text{ K}$. The X-ray observations⁴ suggest that the black-body temperature $T_6 \approx 1$. The optical flux can also originate here; the precise optical-to-X-ray luminosity ratio depending on the observing windows but could be $\ll 10^{-3}$. Further emission may originate at the secondary and in the beams themselves. The work function of the surface is $\sim 1\text{--}10 \text{ keV}$ at the relevant magnetic field strengths¹⁰, so thermionic emission at the black-body temperature cannot supply the neutralizing charge, although there may be an optically-thin outer region heated to greater temperatures.

The γ -ray modulation is assumed to be due to variation in the magnetic field strength at the base of the common flux tube as it travels over the primary star, caused by misalignment of the rotation and magnetic axes and/or non-dipolar components of the field. The 60-s period is identified as $2\pi/\omega$ (see equation (5)). There are three independent frequencies in this system (two spins and the orbit) which could lead to great complexity in its periodic behaviour. In particular, the frequency of variation may be different in different wavebands, so that the period derivative from Bignami *et al.*⁵ could even be incorrect.

In order that $P_m \approx P_R$, the orbital separation of the two neutron stars, a , must be $\leq 10^8 \text{ m}$. If they are each of $1 M_\odot$, then the orbit will decay due to gravitational radiation in $\leq 30,000 \text{ yr}$. The power radiated by gravitational waves is $\approx 3 \times 10^{29} a_8^{-5} \text{ W}$ and exceeds that as electromagnetic radiation. In this model, Geminga is essentially a 'gravity wave binary'. The relative strain at Earth is $\approx 6 \times 10^{-20} a_8^{-1}$ and is unfortunately as yet undetectable¹⁴.

In view of the short anticipated life of Geminga, both to spin-down ($\sim 800 \text{ yr}$) and to gravitational radiation ($\approx 30,000 \text{ yr}$), it is important to consider its formation. Binary neutron stars may form in the Galaxy at a rate¹⁵ of $\sim 3 \times 10^{-4} \text{ yr}^{-1}$ with orbital periods of between one and a few hours. The *a priori* probability of the existence of such a neutron star binary within $\sim 100 \text{ pc}$ approaches one, as the lifetime to gravitational radiation is $\sim 10^7\text{--}10^8 \text{ yr}$. To observe it during a phase lasting $\leq 10^3 \text{ yr}$ and at a distance of $\sim 10 \text{ pc}$, however, makes Geminga exceptional.

Beaming of the γ -ray flux can relax the distance estimate. We also note that any model involving a rapidly-spinning neutron star in which the 60-s period is due to free precession and which spins down in $< 10^3 \text{ yr}$ dissipates $> 10^{30} \text{ W}$. It is then difficult to see how Geminga could be at a distance of $< 100 \text{ pc}$. A possible alternative interpretation in which the object is nearby would be that the $\sim 800 \text{ yr}$ time scale is due to change in distortion of such a pulsar with a weak magnetic field and spin period of $\sim 100 \text{ ms}$. The free-precession frequency is proportional to the difference between the principal moments of inertia. It is then a mystery as to why Geminga is not a radio pulsar⁴. The object would also be a strong source of gravitational waves.

After formation, a neutron star binary goes through a pulsar phase until the speed-of-light cylinder of the star with the greater magnetic moment passes beyond the orbital separation that is

$$F \leq c/a \quad (8)$$

After this phase the unipolar generator mechanism will take over control of the spin-down rate, so that from equation (6)

$$\frac{dF}{dt} = -\frac{4\sqrt{3} \pi \alpha^2 \mathcal{M}^2 (F - \sqrt{GM/a^3})}{\mu_0 \mathcal{J} F a^3} \quad (9)$$

where $\mathcal{J}$ is the moment of inertia of the primary. The spin-down has an insignificant effect on the orbit (because the orbital angular momentum always greatly exceeds that in the spin), so that the orbit decays due to gravitational radiation¹⁶ as

$$a = \left[a_0^4 - \frac{256 G^3 M_1 M_2 M}{5 c^5} t \right]^{1/4} \quad (10)$$

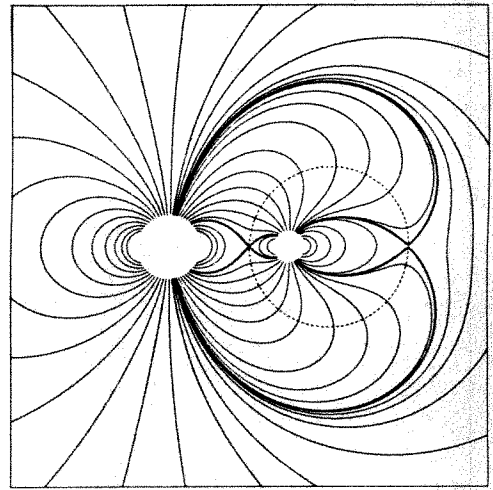


Fig. 1 Magnetic field of a pair of opposed coplanar dipoles. This shows some field lines (not equally spaced in flux) in the plane containing the two dipoles, for $\alpha = 0.5$ (see equation (1)). The boundary of the common flux tube, the 'last common field lines', is shown as bolder than the rest. The dashed circle separates the regions dominated by the two dipoles. The magnetic field is zero on a circle surrounding the weaker dipole where the last common field lines cross over. Although distorted, this feature remains when the dipoles are (moderately) misaligned and when currents $\leq I_{\max}$ flow. There are large potential differences across the boundary of the common flux tube, particularly near the cross-over points where the two regions of isolated field come into contact.

from the initial separation a_0 . While the spin frequency, $F \gg$ the orbital frequency, $\sqrt{GM/a^3}$, equations (9) and (10) may be combined to give

$$(1 - F/F_0) \approx \Gamma(1 - a/a_0) \quad (11)$$

where F_0 is the initial spin frequency, and

$$\Gamma \approx 210 \alpha^2 \chi^2 / \xi a_0^2 \quad (12)$$

where $a_0 = 10^9 a_9 \text{ m}$, $F = \xi c/a_0$ (compare with equation (8)), the polar magnetic field on the primary is

$$B_1 = \chi m_e^2 c^2 / \hbar = 4.7 \times 10^9 \chi \text{ T} \quad (13)$$

and the primary and secondary stars have both been given the parameters adopted above. The remarkable feature of equation (12) is that for reasonable values of the parameters $\Gamma \sim 1$. The binary orbit thus decays at a comparable rate to the spin of the primary. Failing this condition, either the orbit would decay without any significant spin-down ($\Gamma \ll 1$; mostly spin-up) or the spin-down would bring the primary close to corotation before the orbit decayed significantly ($\Gamma \gg 1$). (It is possible that the system did start with $\Gamma \ll 1$, but in that case the gravitational decay time now must be very short indeed, $\ll 800 \text{ yr}$, and the primary star must have started with a spin period $\approx 60 \text{ s}$.) The required high magnetic moment of the primary neutron star implies that it cannot be very old ($\leq 10^7 \text{ yr}$) if current ideas¹⁷ on the decay of magnetic fields are correct.

There is an alternative to the above evolutionary picture which can also produce the Geminga system. The unipolar generator is self exciting once a substantial current has been established because this heats the foot of the flux tube as discussed above. However, if there is very little matter in the magnetosphere the currents may not be able to turn on spontaneously. Perhaps the event¹⁸ of AD 437 was associated with the start of the current flow, caused by accretion of matter passing through the system or by a crustquake¹⁹.

The final outcome of the close neutron star binary we have proposed for Geminga is uncertain²⁰, but it could be a black hole or a millisecond pulsar¹⁵.

We thank K. A. Arnaud, G. F. Bignami, D. O. Gough, E. Priest, J. E. Pringle and M. J. Rees for discussions.

Received 5 July; accepted 22 August 1984.

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Is Cygnus X-3 a monoenergetic 10^{17} eV accelerator?

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Like many other X-ray sources, Cygnus X-3 seems to be a binary system that includes a compact object, but it is unusual in being enshrouded in gas, in displaying occasional huge outbursts and in emitting much more energy above 10^{11} eV than in the keV X-ray region. If the compact object is a pulsar, the ultra-high-energy photons can be products of an accelerated beam of protons (or nuclei) striking the companion's atmosphere^{1,2}. It is noted here that a roughly monoenergetic spray of $\sim 10^{17}$ eV protons at $\sim 10^{39}$ erg s⁻¹, generating an electron-photon cascade in the environs of the companion star, best accounts for all photons above a few GeV, and might generate the gas shroud, as beam energy deposition inside the star could lift off a thick layer of matter to generate a fountain under the pulsar. This evidence on the nature of the accelerated beam is particularly significant as Cygnus X-3 seems likely to be responsible for most of the cosmic ray particles around 10^{17} eV which are at present being injected into the Galaxy.

γ Rays are seen briefly at two portions of the 4.8-h binary period, at phases reported variously as 0.15–0.25 and 0.63–0.8, taken from the time of deepest eclipse of the X-ray source; the spectrum extends (roughly as $E^{-2} dE$, where E represents energy) to 10^{16} eV (refs 3, 4). Vestrand and Eichler^{1,2} have suggested that we see the pulsar through the tenuous outer layers of the companion just before and after eclipse, and see photons from nuclear interactions (via π^0 decay) in the gas. This is supported by the finding that although a potential drop of 10^{17} V required to accelerate charged particles could hardly be found more than ~ 100 km from the pulsar, the 10^{16} eV photons cannot penetrate a transverse magnetic field exceeding 500 G (ref. 5) and hence must be formed far away, somewhere near the companion star ($\sim 5 \times 10^{10}$ cm distant), where the field is believed to be of this magnitude^{5,6}. However, three problems arise: (1) The diameter of the relevant outer surface of the companion must be very large¹, as it appears to eclipse the source of X rays and low-energy γ rays for $\sim 40\%$ of the orbit. Modelling of the intensity curves⁷ indicates that the shadow must be cast by gas present far outside any plausible Roche lobe of the companion unless the pulsar is extraordinarily light. (2) How is the spectrum generated? (3) How do the 10^{12} eV photons remain well collimated if they are derived from 10^{13} eV protons, as has generally been supposed? To explain radio emission, Vestrand⁶ requires a magnetic field (B) of 500–1,000 G to be embedded in the gas, and Stephens⁴ showed that such a field could cause the sharp end to the photon spectrum. The radius of gyration of 10^{13} eV protons would then be less than 10^{-2} of their path either before or after entering the gas, destroying all directionality. I will initially assume that this local field is present, but even if instead the only field arises from the pulsar, one still expects deflection

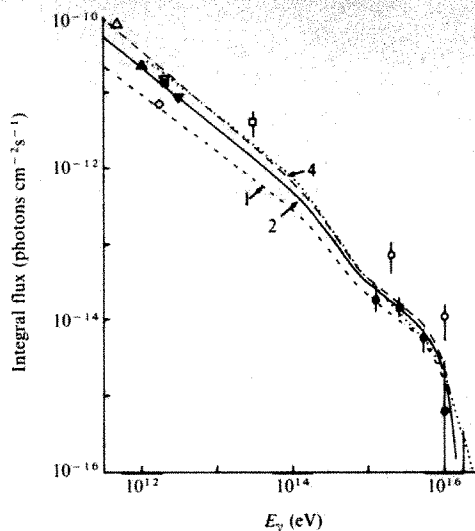


Fig. 1 Calculated photon spectrum resulting from cascades initiated by 10^{17} eV protons in gas surrounding the companion star (magnetic field present), for cases where integration extends to maximum gas thickness 1, 2 and 4 radiation lengths. Magnetic pair production⁵ causes the drop at 10^{16} eV. The dip starting at 10^{14} eV is due to pair production on primeval photons, assuming 12 kpc distance. The dotted line represents a non-magnetic cascade, initiated by 5×10^{16} eV protons (integrated to a maximum thickness of 16 radiation lengths). Data: Δ , ref. 19; $\blacktriangle$, ref. 20; $\diamond$, ref. 17; $\blacksquare$, ref. 18; ∇ , ref. 21; $\blacktriangledown$, ref. 22; $\square$, ref. 23; $\circ$, ref. 3; $\bullet$, ref. 4.

of protons from their regular streaming when this regular guiding field is distorted on meeting the gas.

As the pulsar enters eclipse, we must see it through successively greater thicknesses of gas, and hence should see an electron-photon cascade emerging. Figure 1 shows the time-averaged photon spectrum generated by a monoenergetic 10^{17} eV proton beam, assuming that the duration of viewing through different column densities μ of gas varies as μ^{-1} (as in an exponential atmosphere), although this is not critical. The density is typically $\sim 4 \times 10^{-9}$ g cm⁻³, and if there is an embedded field > 10 G, the cascade develops by alternate pair production and very rapid synchrotron radiation rather than by normal bremsstrahlung (and there is little contribution from column densities exceeding 2–4 conventional bremsstrahlung radiation lengths of ~ 40 g cm⁻²). A photon of energy E_γ eV is typically radiated by an electron of energy $E_e = 5.5 \times 10^9 (E_\gamma/B)^{1/2}$ eV (B in gauss). (Alternatively, without a local magnetic field, normal bremsstrahlung dominates: the ultimate spectrum and energy requirements are virtually the same, but more radiation lengths are required, partly because E_γ then falls as E_e rather than E_e^2 . The dotted line in Fig. 1 shows how the photon spectrum then terminates, for monoenergetic 5×10^{16} eV protons.) By choosing the flux of 10^{17} eV protons to fit the photon flux⁴ around 10^{15} eV, it accounts for the 10^{12} eV photons also: there is little room for additional production by protons below 10^{16} eV. With 10^{17} eV protons and a synchrotron cascade, there is no collimation problem. The 10^{12} eV photon is radiated by a 2×10^{14} eV electron (if $B = 500$ G) which suffers negligible deflection in travelling its typical radiation mean free path $10^7/(E_{14}B^2)$ cm, and the direction of the original 10^{17} eV proton is maintained. (There will, nevertheless, be a few widely spread photons, arising from muons which decay after many magnetic gyrations.)

The accelerated beam need not be strictly monoenergetic; we require the energy carried by protons above 5×10^{16} eV to be greater than that carried by protons in the four decades of energy below this, which can also contribute to the TeV photons, and the beam energy could be higher than 10^{17} eV, especially if hadron interactions greatly change⁸ at energies beyond the range explored by accelerators and fitted by the interaction model. Nuclei of the same energy per nucleon are permitted.

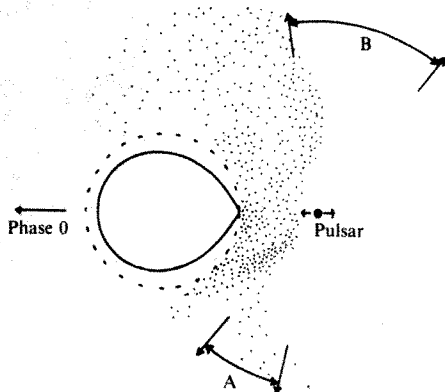


Fig. 2 Binary system, showing approximate directions to the observer when γ -ray bursts are observed (A, phase ~ 0.2 ; B, phase ~ 0.75), and fountain of ejected matter.

To give the flux shown in Fig. 1, the energy in the proton beam above 10^{16} eV must be $2 \times 10^{39} (\Omega/4\pi)(0.05/\Delta) (D/12 \text{ kpc})^2 \text{ erg s}^{-1}$, where D is the distance to Cygnus X-3, Ω is the solid angle into which the overall proton beam is emitted, and Δ is the duty cycle of one photon pulse at high energies (which may be as small as 0.02 (refs 3, 4)). Thus, the power L_p in the particle beam is about $10^{39} \text{ erg s}^{-1}$. Most of the protons (or nuclei) will escape into the Galaxy, providing $\sim 10^{39} \text{ erg s}^{-1}$ above 10^{16} eV per nucleon, and little of significance below this energy.

Wdowczyk and Wolfendale⁹ have already pointed out that about 30 Cygnus X-3s might supply the power to maintain the Galaxy's cosmic rays; but in the energy range above 10^{16} eV, one finds a more remarkable situation. How much power is required to maintain the galactic cosmic rays above 10^{16} eV against leakage? A transport model¹⁰ indicates a lifetime in the galactic disk (say 15 kpc radius and 0.6 kpc thick) of around $2.5 \times 10^5 \text{ yr}$ at this energy, which is also compatible with the lifetime of $>10^6 \text{ yr}$ at 10^{14} eV suggested by Kiraly *et al.*¹¹. Their local energy density $\sim 3 \times 10^{-17} \text{ erg cm}^{-3}$ would then require a power of $\sim 5 \times 10^{37} \text{ erg s}^{-1}$. Alternatively, one can use the variation of anisotropy with energy to estimate¹² the energy spectrum emerging from galactic sources to be $\sim E^{-2.47} dE$ particles s^{-1} (rather like the lower-energy source spectrum $E^{-2.44} dE$ of heavier nuclei deduced from spallation¹³). In this case, $\sim 5 \times 10^4$ of all injected cosmic ray energy goes into the particles above 10^{16} eV, that is, $2.5 \times 10^{37} \text{ erg s}^{-1}$. Hence, the Galaxy's needs are evidently $\leq 10^{38} \text{ erg s}^{-1}$ averaged over 10^5 yr , and sources like Cygnus X-3 can be present only for a part of this time interval. It is therefore unlikely that any other such objects exist in the Galaxy. (The source Vela X-1 is only $\sim 10^{-2}$ as powerful¹⁴.) If the source emits a monoenergetic spectrum, it is normally assumed that one must rely on evolution of beam energy with time to generate the observed spectrum of galactic cosmic rays¹⁵. Cygnus X-3 might, of course, be less powerful than assumed if its pulsar is an aligned rotator, only firing its protons towards us (small solid angle Ω).

Where it strikes the star directly, the beam will deposit $\sim 40\%$ of its energy in an electron-photon cascade at depths 100–500 g cm^{-2} . Here, at $z \sim 100$ Thomson scattering mean free paths below the surface, the thermal leakage time is long, and it will generate a pressure $0.5 L_p z / 4\pi d^2 c$ (if the absorbing layer is at a distance d from the pulsar), z times the direct pressure of the beam. This will easily lift off the overlying few hundred g cm^{-2} if $M_{\text{star}} \sim (0.3-3) M_{\odot}$, and should be far more effective than keV X-ray absorption in ejecting matter. (The mechanical work done in removing $2 \times 10^{-3} M_{\odot} \text{ yr}^{-1}$ —taken as an upper limit⁶—is $< 10^{38} \text{ erg s}^{-1}$.) Nearby surface matter must therefore be lifted off, and a fountain of gas under the pulsar may normally cushion the main body of the star from catastrophic ejection. Although the gas dynamics will be complex¹⁶, a rough idea of the possible disposition of gas is suggested in Fig. 2, based on trajectories of independent particles emitted at up to the escape velocity,

and influenced by a slightly super-eddingtonian radiation pressure from the pulsar. Rotation of the system is responsible for the asymmetry, and the pattern roughly reflects the actual direction from which γ -ray pulses are reported. The large size of the occulting body may arise in this way. Dowthwaite *et al.*¹⁷, however, report a 10^{12} eV signal at phase as early as 0.63, when the pulsar is presumably well in front of the companion. A small X-ray spike is often seen at this point, and as the pulsar is probably plunging in towards periastron near here⁷, it may become embroiled with the gas at this moment. The 34-day variation may be related to a lag in rotation of the companion, along with its magnetic dipole, as this may affect the gas flow.

A requirement of this model is that the 10^{15} eV γ -ray burst should be slightly offset in time (by about half the burst duration—say 0.02 cycle) from the 10^{12} eV γ rays, which require more matter for their production (TeV photons earlier at the 0.2 phase). This may be the case^{4,18}, but there has been uncertainty in the synchronization of phases between different workers, observing over different periods, and it would be desirable to test this by measuring the relative timing of pulses of high and lower energy over the same interval. The 0.7-phase burst may fluctuate somewhat in position, and if so, the start of the sharp intensity drop in X rays should remain synchronized with this γ -ray burst.

Received 5 June; accepted 28 August 1984.

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Rising atmospheric carbon dioxide concentrations may increase streamflow

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Historically, studies of the greenhouse effect of carbon dioxide (CO_2) have dealt primarily with temperature and only secondarily with precipitation. In the latest report on this topic¹, however, the subject of streamflow is broached with an analysis² which suggests that watersheds in the western United States will suffer 40–75% reductions in streamflow for a doubling of the atmospheric CO_2 content, leading to a 2°C rise in air temperature and a 10% drop in precipitation. A shortcoming of that study is that it does not include the direct antitranspirant effect of atmospheric CO_2 enrichment that would accompany any CO_2 -induced climatic change, whereby increasing CO_2 content of the air tends to induce partial stomatal closure, so reducing plant transpiration and thereby

conserving soil moisture and increasing runoff to streams. Inclusion of this latter effect in a simple model of watershed runoff applied to 12 drainage basins in Arizona indicates that 40–60% increases in streamflow may well be the more likely consequences of a CO₂ concentration doubling, even in the face of adverse changes in temperature and precipitation.

To ensure valid comparability with the National Research Council (NRC) study¹, we used the identical model employed by them to estimate changes in runoff induced by changes in temperature and precipitation and then superimposed the anti-transpirant effect of atmospheric CO₂ enrichment on those results. Thus, we will first briefly describe that model. Table 1 represents a set of empirical relationships among temperature, precipitation and runoff derived by Langbein *et al.*³ for relatively arid areas in the western United States. Our first step was to confirm that this tri-factor matrix used by Revelle and Waggoner² did indeed apply to the 12 Arizona drainage basins we selected for study, which are described in detail elsewhere⁴. Thus, we first derived mean annual values of surface air temperature and precipitation from a report⁵ which gave this information at grid points distributed at spacings of 16 km, and used the results to calculate watershed runoff by this method. Then, from another report⁶ which gave the long-term (30–50 yr) mean annual runoff of the 12 drainage basins as directly measured by stream gauges, we extracted data to use as a standard for comparison. This exercise yielded the plot of Fig. 1, where it can be seen that the Langbein model does indeed adequately describe the mean annual runoff of the 12 Arizona drainage basins selected for study.

We next used the Langbein model of Table 1 in a manner analogous to that of the NRC study to determine the effects of possible CO₂-induced changes in climate. The scenarios we considered were: first, the same 2 °C rise in temperature and 10% drop in precipitation investigated by the NRC report authors; second, a 2 °C rise in temperature with no change in precipitation; and third, a 10% drop in precipitation with no change in temperature. Mean percentage decreases in streamflow for the five wettest basins derived for these scenarios were, respectively, 41, 23 and 23%, with some of the drier basins actually suffering 100% reductions and ceasing to provide any streamflow whatsoever. Up to this point all the results accord with those of Revelle and Waggoner².

Following the lead of the NRC report, we next expressed

Table 1 Mean annual runoff (mm yr⁻¹) as a function of mean annual temperature (°C) and mean annual precipitation (mm yr⁻¹) for the arid southwestern United States

Temperature	Precipitation					
	200	300	400	500	600	700
-2	54	92	154	230	330	440
0	40	74	124	190	275	380
2	28	57	95	154	225	320
4	17	40	78	125	190	265
6	9	25	60	100	155	220
8	0	17	42	82	128	185
10		8	29	64	103	155
12		0	19	47	80	130
14			10	32	65	105
16			0	20	50	85

Sources: refs 2, 3.

runoff (R) as the difference between precipitation (P) and evapotranspiration (E), setting $R = P - E$. Knowing R and P , we could thus solve this equation for E for current conditions, as well as for the three climate change scenarios previously described, and for each of the 12 drainage basins. Then, making use of the experimentally-documented fact that plant evaporative water loss is generally reduced to about two-thirds of its original rate by a doubling of the atmospheric CO₂ content^{7,8}, we adjusted each of these evapotranspiration results to reflect what they would be with the antitranspirant effect of atmospheric CO₂ enrichment superimposed on them. In doing this, however, it was necessary to express the adjusted evapotranspiration (E_a) as $(1 - 1/3 f)E$, where f represents the fractional area of the drainage basin covered by vegetation, as none of the basins was completely covered with plants. Thus, if there were no vegetation ($f = 0$) there would be no vegetative effect and E_a would equal E , whereas if the entire watershed were covered with vegetation, ($f = 1$), E_a would equal $2/3 E$. Our evaluations of f for this purpose were made from detailed descriptions and photographs of the 12 basins associated with the most recent vegetation maps of the area⁹. Values of f obtained by this method varied from a low of 0.24 to a high of 0.61, with an average for the five wettest basins of 0.49.

Following this procedure to obtain E_a values for each of the

Table 2 Mean annual observations and calculations of runoff for the five wettest basins studied for several climate change scenarios

Basin no.	Vegetation fraction	Observations			Calculated runoff							
		Mean annual T (°C)	Mean annual P (mm)	Mean annual R (mm)	Without vegetation effect			With vegetation effect				
					+2°-10% (mm)	+2°-0% (mm)	+0°-10% (mm)	+2°-10% (mm)	+2°-0% (mm)	+0°-10% (mm)	+0°-0% (mm)	
4906	0.61	9.3	627	111	78	101	105	177	203	198	215	
4940	0.57	9.3	607	105	69	91	97	160	188	183	200	
4985	0.42	11.3	559	70	46	56	53	109	126	115	138	
4990	0.41	12.6	588	74	38	49	58	105	129	122	143	
5053	0.45	11.9	562	108	43	62	51	112	128	119	176	
Mean	0.49	10.9	589	93	55	72	72	132	155	147	174	

Basin no.	% Change from observed							
	Without vegetation effect			With vegetation effect				
	+2°-10%	+2°-0%	+0°-10%	+2°-10%	+2°-0%	+0°-10%	+0°-0%	
4906	-30	-9	-5	+59	+83	+78	+94	
4940	-34	-13	-8	+52	+79	+74	+90	
4985	-34	-20	-24	+56	+80	+64	+97	
4990	-49	-34	-22	+42	+74	+65	+93	
5053	-60	-43	-53	+4	+19	+10	+63	
Mean	-41	-23	-23	+42	+67	+58	+87	

+2°-10% = 2 °C rise in temperature with a 10% reduction in precipitation.

+2°-0% = 2 °C rise in temperature with no change in precipitation.

+0°-10% = no change in temperature with a 10% reduction in precipitation.

+0°-0% = no change in either temperature or precipitation.

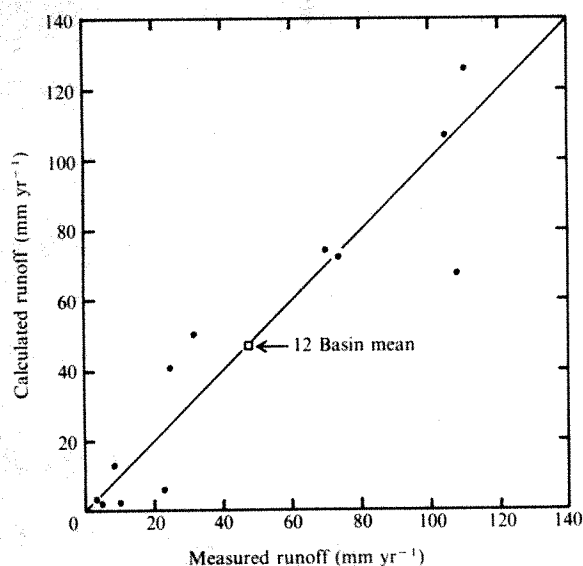


Fig. 1 Mean annual runoff from 12 Arizona drainage basins calculated by the Langbein model of Table 1 compared with measured mean annual runoff.

12 basins for the four scenarios under consideration (three climate change scenarios and one no-change situation), we next used these results to evaluate adjusted runoff (R_a) values which incorporated both the effects of the selected climatic changes (or non-change) plus the direct antitranspirant effects of a CO_2 concentration doubling. The results of this process, summarized in Table 2, indicated that for the first climate change scenario, that is, the 2°C rise in temperature and 10% drop in precipitation predicted in the NRC study, the mean change in streamflow of the five wettest basins calculated to accompany a doubling of the atmospheric CO_2 content went from -41% to $+42\%$ when the antitranspirant effect of the atmospheric CO_2 enrichment was added to the climate change effect. Similarly, when the vegetative effect of CO_2 was added to the climate change effect of the second scenario—a 2°C rise in air temperature with no change in precipitation—the mean change in streamflow of the five wettest basins went from -23% to $+67\%$. And for the third scenario—a 10% drop in precipitation with no change in temperature—it went from -23% to $+58\%$; while for the case of a negligible CO_2 effect on climate, it went from the status quo to $+87\%$. In all of these situations, we note that streamflow increases for the drier basins were of the order of several hundred percent.

We note also that our results are comparable with those recently obtained by Aston¹⁰ with a distributed deterministic process model used to simulate the effects of changed stomatal resistance on streamflow in Australia. Indeed, Aston concluded that “we can expect streamflow to increase from 40 to 90% as a consequence of doubling the atmospheric CO_2 concentration”, in what could also be a statement of our conclusions.

There are several simplifications and uncertainties associated with the above analysis which preclude its being accepted as the final word on the subject. For one thing, the fractional area of vegetation (f) is difficult to assess very accurately. In addition, it could well increase significantly in a CO_2 -enriched world¹¹, and it is likely that an unvegetated area will provide more runoff than a vegetated area of equal size. Also, a small infiltration component of the total water balance is neglected in both our analysis and that of Revelle and Waggoner². Both studies additionally suffer from lack of monthly resolution, and both fail to account for shifts in the time of major watershed runoff which may (or may not) occur as climate changes (or remains the same¹²). However, because both studies have about the same degree of complexity, the very opposite results which they produce illustrate that we are still at the stage where plausible additions to state-of-the-art model studies of the CO_2 ‘problem’

can dramatically alter current preceptions of future consequences.

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Nd evidence for Proterozoic crustal development in the Belt–Purcell Supergroup

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A knowledge of the chemical and isotopic characteristics of the continents as they existed in the past is essential to an understanding of their evolution^{1–7}. Vestiges of these characteristics remain in the sedimentary record. For example, the character and crustal residence age⁴ of ancient continental crust has been deduced from the Sm–Nd isotopic patterns of clastic sediments that have survived to the present day^{4,6,8}. We report here Sm–Nd isotopic measurements from the Belt–Purcell Supergroup of western North America which form the first detailed isotopic investigation of a major Proterozoic sedimentary succession. The Belt–Purcell has many features also seen in the detrital load of the world’s major contemporary rivers, including exceptionally homogeneous $^{147}\text{Sm}/^{144}\text{Nd}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios and a small range of crustal residence ages. Mantle additions to the continental crust seem to have been relatively minor since the middle Proterozoic.

The Belt–Purcell Supergroup of the northwestern United States and southwestern Canada is the most extensive and least disturbed succession of Proterozoic sediments in North America. It outcrops over an area greater than 125,000 km^2 in western Montana, northern Idaho, southwestern Alberta and southern British Columbia (Fig. 1), and lies on ≥ 1.7 Gyr crystalline basement^{9,10}. Shallow water structures predominate throughout the succession^{11–15}. The time of deposition of the supergroup is uncertain, but may have spanned an interval of 1,500–1,200 Myr^{10,16–18}. Unconformably overlying the Belt–Purcell Supergroup are the impure clastics of the Windermere Supergroup, deposited around 800–600 Myr^{15,19}.

Proposed explanations for the depositional environment of the Belt–Purcell Supergroup include deposition in a miogeocline or within an adjacent failed arm of a rift system, and suggestions that Belt–Purcell deposition coincided with incipient rifting of a western continent, of entirely epicratonic deposition^{15,20–24}. Sediment source areas have been tentatively identified to the east, south and south-west of the Belt–Purcell deposit^{12,15,25,26}. However, Mesozoic listric thrust faulting within the Belt–Purcell basin involved translations of up to 180 km ¹¹ and thus hampers attempts to elucidate the original basin configuration, sediment

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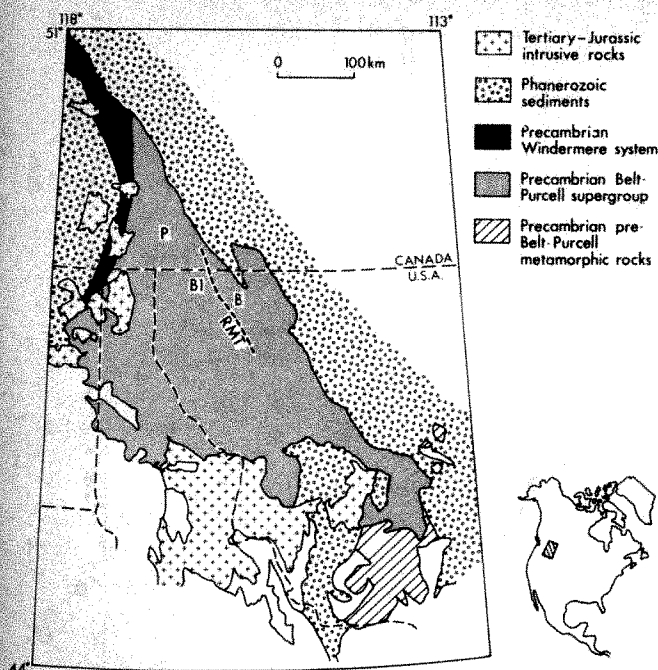


Fig. 1 Geological sketch map showing the location of the Belt-Purcell Supergroup, principal structures, and sample localities. Modified from King³⁴. RMT, Rocky Mountain Trench; B, B1, Belt Series; P, Purcell Series.

source directions or source areas. Information from Sm-Nd isotopes obtained from Belt-Purcell sediments may provide new insights into these problems, as well as into Proterozoic crustal development.

Samples have been selected mainly from two localities, one in the Purcell Mountains, British Columbia, and the other from the vicinity of the Whitefish Range, Montana (Fig. 1). We also analysed a sample from the Horseshoe Creek Group of the Windermere Supergroup. Exact sample locations and stratigraphic positions are described elsewhere²⁷.

Table 1 gives Sm-Nd isotopic measurements on these samples. Analytical procedures used follow those reported previously²⁸. Present-day $^{143}\text{Nd}/^{144}\text{Nd}$ ratios for Belt-Purcell rocks fall within a restricted range of 0.511615 ± 18 to 0.512040 ± 22 ($\epsilon_{\text{Nd}} = -20$ to -12) and $^{147}\text{Sm}/^{144}\text{Nd}$ ratios vary only from 0.099 to 0.120, even though Nd and Sm concentrations vary by a factor of 3–4. These $^{147}\text{Sm}/^{144}\text{Nd}$ ratios are typical of those reported for modern river sediments²⁹ and other fine-grained clastic sedimentary rock^{5,6,8}.

The uniformity of $^{143}\text{Nd}/^{144}\text{Nd}$ ratios for sediments deposited throughout a ~20-km thick succession during a period that may extend over some 300 Myr is striking (Fig. 2a). This constant isotope ratio probably indicates the presence of a particularly homogeneous and persistent sediment source, and/or very thorough mixing of a number of source materials over a period of several hundred million years. Crustal residence ages, which are calculated on the assumption that the Sm/Nd ratios of the sediments are unchanged from the time that their precursory continental material was generated by magmatic processes, are also uniform for the Belt-Purcell sediments, and with one exception fall between 1.9 and 2.1 Gyr (Table 1, Fig. 2b). The mean crustal residence age of the uniform source of sediment is well in excess of the depositional age and new mantle additions to the continent within the Belt-Purcell source area, over the interval concerned, are too small to be recognizable. The one sample which possesses a somewhat younger crustal residence age of 1.6 Gyr presumably reflects a larger than average additional contribution from a relatively young and probably locally supplied source.

It is useful to compare crustal residence and depositional ages of Belt-Purcell sediments with published results for Archaean sediments. The crustal residence age for Belt-Purcell samples

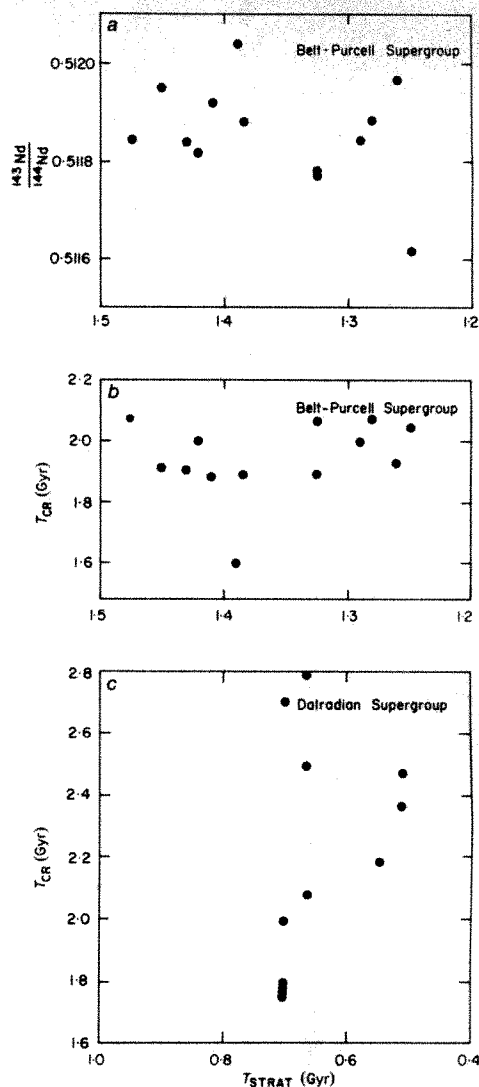


Fig. 2 a, $^{143}\text{Nd}/^{144}\text{Nd}$ plotted with respect to approximate stratigraphic ages for Belt-Purcell sediments. Stratigraphic ages are inferred from palaeomagnetic data¹⁶. b, Crustal residence age estimates (T_{CR}) plotted with respect to approximate stratigraphic age (T_{STRAT}) for Belt-Purcell sediments. c, Crustal residence age estimates (T_{CR}) plotted with respect to approximate stratigraphic age (T_{STRAT}) for Dalradian Supergroup samples. Comparison of b and c illustrates that whereas the Belt-Purcell shows a small range in crustal residence age throughout a 20-km succession, the Dalradian shows an unusually wide variation in crustal residence age. (See also ref. 35.)

exceeds the depositional age by 200–800 Myr, whereas available Sm-Nd analyses of Archaean sediments from Isua, Malene, Pilbara and Fig Tree deposits indicate crustal residence ages approximately coincident with their stratigraphic ages^{4,6,8,28}. This result has been interpreted to indicate that these Archaean sediments are close to first-cycle⁴, because short time intervals around 100 Myr have elapsed between extraction of parent material from the mantle and deposition of the sediment. The contrasting character of the Archaean and Belt-Purcell sediments indicates that the latter has a longer average time interval between crustal formation and sediment deposition and possibly a more extensive history of recycling. The Windermere Supergroup sample (Table 1) has a lower $^{143}\text{Nd}/^{144}\text{Nd}$ ratio (0.51154) than that of the Belt-Purcell samples, and higher crustal residence age of 2.4 Gyr, perhaps reflecting the contribution of a different, older source material or proportion of such material to the Windermere.

The sedimentology of the Belt-Purcell rocks does not constrain their source. However, to evaluate one of several possible source areas, eight Canadian Shield basement samples have

Table 1 Nd and Sm isotope data for fine-grained clastic sediments from the locations within the Belt-Purcell Supergroup

Belt Series: Whitefish Range and vicinity						
Sample	$^{143}\text{Nd}/^{144}\text{Nd}(2\sigma)^*$	Nd (p.p.m.) [†]	Sm (p.p.m.) [†]	$^{147}\text{Sm}/^{144}\text{Nd}$	T_{CR} (Gyr)	T_{STRAT} (Gyr)
B1	0.511842 (20)	24.78	4.814	0.1174	2.07	1.47
B2	0.511836 (18)	36.13	6.639	0.1111	1.95	1.43
B3	0.511917 (16)	44.22	8.329	0.1139	1.88	1.41
B4	0.512040 (22)	41.19	7.396	0.1072	1.59	1.39
B5	0.511771 (20)	47.41	8.799	0.1122	2.07	0.32
B6	0.511965 (14)	28.45	5.665	0.1204	1.93	1.26
Purcell Series: Purcell Mountains and vicinity						
P1	0.511950 (18)	42.21	8.238	0.1180	1.91	1.45
P2	0.511814 (18)	44.86	8.219	0.1107	1.97	1.42
P3	0.511879 (18)	32.16	5.891	0.1107	1.88	1.38
P4	0.511764 (15)	38.26	6.460	0.1021	1.89	1.32
P5	0.511842 (18)	52.93	10.04	0.1146	2.01	1.29
P6	0.511882 (24)	58.98	11.76	0.1205	2.07	1.28
P7	0.511615 (18)	20.40	3.348	0.0992	2.04	1.25
Windermere Supergroup: Purcell Mountains and vicinity						
P8	0.511537 (20)	36.04	6.543	0.1097	2.63	<0.750

$^{143}\text{Nd}/^{144}\text{Nd}$ ratios are normalized to $^{146}\text{Nd}/^{144}\text{Nd} = 0.7219$. Bulk Earth parameters used to calculate ϵ_{Nd}^T are $^{143}\text{Nd}/^{144}\text{Nd} = 0.512638$ and $^{147}\text{Sm}/^{144}\text{Nd} = 0.1966$. Crustal residence ages (T_{CR}) are calculated relative to a depleted mantle where $\epsilon_{\text{Nd}}^{4.6} = 0$ and $\epsilon_{\text{Nd}}^0 = +10$ (ref. 29). Samples are numbered B1 to B6 and P1 to P7 from the base to the top of the stratigraphic succession collected from the Belt and Purcell Series respectively. T_{STRAT} ages are approximate.

* Errors quoted are 2 standard deviations of the mean.

† Error $\leq 0.2\%$.

been obtained from cores in southern British Columbia and Alberta recovered from depths of 2,200–3,700 m. The results for these samples (Table 2) are compared with previous Nd isotopic analyses reported from the Canadian Shield⁸. Crustal residence ages for the eastern and western parts of the shield are similar and range respectively over 2.6–3.2 and 2.8–3.4 Gyr, with the one exception of a Grenville composite which has a crustal residence age of 1.5 Gyr. No part of the Canadian Shield has yet been identified which has a 2.0 Gyr crustal residence age such as might have acted as a single eastern sediment source to the Belt–Purcell, and furthermore, no mixture of these Canadian Shield regions west of the Grenville Province yields an average crustal residence age of 2.0 Gyr. If multiple sources with a range of crustal residence ages are involved, they must have become effectively homogeneous by the time of deposition.

The range in crustal residence ages for both modern river particulates²⁹ and Belt–Purcell sediments suggests that the Belt–Purcell material, like modern river sediment, is the product of

effective homogenization accompanying fluvial processes. Lithofacies evidence of braided stream, alluvial fan and deltaic deposits supports this notion, as does the general uniformity of sediment mineralogy and geochemistry^{12,23,26,30}. By analogy with modern major river systems, the Belt–Purcell succession may be the product of erosion across a large drainage area, and sediment sources may not have been restricted to proximal areas.

It should be emphasized that the particularly restricted range of crustal residence age estimates found for the Belt–Purcell is not a feature of all sedimentary successions. For example, the Dalradian Supergroup of Scotland, for which considerable data are available, exhibits very different characteristics (Fig. 2c)^{4,31}. The Dalradian was deposited with an interval extending over 0.70–0.51 Gyr, and shows a very wide range of crustal residence ages (1.7–2.8 Gyr). These results are consistent with localized and heterogeneous source regions, accessed by drainage systems of restricted areal extent. The Dalradian results provide an

Table 2 Nd and Sm isotope data for Canadian Shield samples

New data from basement cores, Southern British Columbia and Alberta					
Sample	$^{143}\text{Nd}/^{144}\text{Nd}(2\sigma)^*$	Nd (p.p.m.) [†]	Sm (p.p.m.) [†]	$^{147}\text{Sm}/^{144}\text{Nd}$	T_{CR} (Gyr)
UA4221	0.511275 (26)	4.125	0.7736	0.1133	2.83
UA4229	0.511001 (18)	59.02	8.702	0.0891	2.62
UA4418B	0.511135 (20)	18.37	3.263	0.1074	2.87
UA4220	0.511448 (24)	21.64	4.065	0.1135	2.58
UA2438	0.510381 (14)	49.51	4.955	0.0610	2.75
	0.510363 (14)				
UA4231D	0.511258 (17)	14.31	2.934	0.1239	3.19
UA3185	0.511217 (18)	47.75	8.615	0.1091	2.80
UA5282	0.510979 (16)	32.92	5.351	0.0982	2.85
Previously reported data for Canadian Shield composites ⁸					
Sample	$\epsilon_{\text{Nd}}^{(0)}$	$f_{\text{Sm}/\text{Nd}}^{\ddagger}$	T_{CR} (Gyr) [§]		
New Quebec	-29.4 ± 0.4	-0.444	2.94		
North Quebec	-28.9 ± 0.3	-0.464	2.79		
Fort Enterprise gneiss	-24.3 ± 0.4	-0.372	3.37		
Fort Enterprise granite	-30.7 ± 0.2	-0.495	2.77		
Baffin Island	-31.7 ± 0.3	-0.464	3.00		
Saskatchewan	-32.5 ± 0.4	-0.486	2.94		
Quebec	-7.1 ± 0.3	-0.361	1.52		

* Errors quoted are 2 standard deviations of the mean.

† Error $\leq 0.2\%$.

$\ddagger f_{\text{Sm}/\text{Nd}} = \frac{(\text{Sm}/\text{Nd})_{\text{sample}}}{(\text{Sm}/\text{Nd})_{\text{CHUR}}} - 1$.

$\S T_{\text{CR}}$ calculated from $\epsilon_{\text{Nd}}^{(0)}$ and $f_{\text{Sm}/\text{Nd}}$ data of McCulloch and Wasserburg⁸.

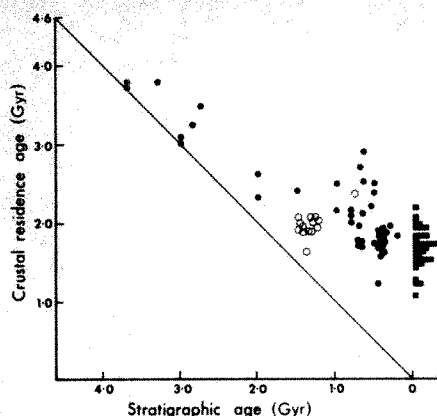


Fig. 3 Comparison of the stratigraphic ages and crustal residence ages of fine-grained clastic sediments calculated from Sm-Nd isotopic analyses from this study and elsewhere^{4,6,8,29,31}. Additions to the sedimentary mass from contemporary mantle will plot near the line indicated. Open circles, Belt-Purcell samples; closed circles, all other samples; closed squares indicate river and aeolian particulates.

excellent example of the value of Sm-Nd isotopic analyses of sediments for limiting the nature of sediment source regions.

A comparison of crustal residence ages for the Proterozoic Belt-Purcell Supergroup and sediments of other depositional ages (Fig. 3) shows that all sediments deposited since 2.0 Gyr have crustal residence ages well in excess of their stratigraphic ages. Assuming that the samples analysed are typical of sediment of their respective stratigraphic ages, it appears that the source areas of sediments deposited before ~2.0 Gyr must include a large component of material with a comparatively short crustal residence time. In contrast, the younger sediments are dominated by recycled continental components with relatively long crustal residence times, and minimal additions of contemporary mantle-derived material. Interestingly, the average crustal residence age for modern river particulates, Palaeozoic sediments and the Belt-Purcell Supergroup are all around 1.7–2.0 Gyr. Although it might be argued that this pattern is an artefact arising from limited sampling and that the Belt-Purcell may be atypical of the global sedimentary mass existing at that time, it seems more likely that the input of new mantle material into the sedimentary mass has been minor over the past 1.5 Gyr or so. Because the evolution of the sedimentary mass is likely to be reasonably representative of at least the upper part of the continental crust, it would appear that the evolution of the continents themselves has also been dominated by recycling.

An important conclusion from the evidence now available is that the mean crustal residence age of clastic sediments, and by inference that of the continental crust during much of the Archaean, differed little from that expected for contemporary additions from the mantle at that time. This observation eliminates the possibility of major contributions from pre-4.0 Gyr continental crust, and suggests that the residence time of light rare-earth enriched material at the Earth's surface was short during this interval^{32,33}. The dominance of material with a short crustal residence age evident in the archaean sedimentary mass is reduced markedly throughout the Proterozoic. Recycling and cannibalism of older continental crust has dominated the Proterozoic and Phanerozoic evolution of the sedimentary mass.

We thank Drs R. A. Burwash, J. Harrison, J. Patchett, Z. Peterman and A. G. Smith for critical reviews. This work has been partially supported by an NSF grant to C.D.F., the Royal Society, and the Natural Environment Research Council. Cambridge Department of Earth Sciences Contribution No. 257.

Received 11 May; accepted 16 July 1984.

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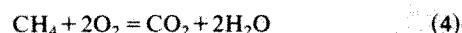
Metamorphism of reduced granulites in low-CO₂ vapour-free environment

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Pervasive flooding of CO₂ has been proposed as the cause of granulite facies metamorphism that is capable of producing many distinctive characteristics of the deep continental crust: reduced water activity, orthopyroxene-bearing assemblages, depletion of large-ion lithophile (LIL) elements, and dehydration^{1–6}. The calculations presented here of C–O–H fluid composition for conditions of granulite facies metamorphism show that oxygen fugacity (f_{O_2}) estimates from many terranes are sufficiently low that the addition of CO₂-rich fluid causes graphite to precipitate. For values of pressure (P) and temperature (T) common to granulites, and with f_{O_2} slightly below the quartz–fayalite–magnetite buffer (QFM), the addition of CO₂ sufficient to grow 10 vol. % orthopyroxene requires the precipitation of 1.5 vol. % graphite. As 0.1 vol. % graphite is readily recognizable, but is not reported in most low f_{O_2} granulites, these rocks have not been flooded by CO₂, and low f_{H_2O} is probably due to extraction of a magma or recrystallization of an already dry rock^{7,8}. Granulite terranes may thus result from a combination of these three processes and the dominance of any one cannot now be demonstrated on a regional basis.

Calculations in the C–O–H system show that there are six important fluid species (H₂O, CO₂, CH₄, CO, H₂, O₂) which can be related by four independent equations^{9–11}.



At known P and T there are eight variables in this system, the six fluid species, the fluid pressure (P_F) and the activity of graphite (α_C). If a free-fluid phase is present, a condition required by CO₂ infiltration, then $P_F = P_{Lithostatic}$ (P_L) and equation (5) can be assumed.

$$P_L = P_{H_2O} + P_{CO_2} + P_{CH_4} + P_{CO} + P_{H_2} + P_{O_2} \quad (5)$$

By assuming a free-fluid phase there are now five equations and seven unknowns (as P_F has been fixed) and by fixing the value of two variables it is then possible to calculate the values of the

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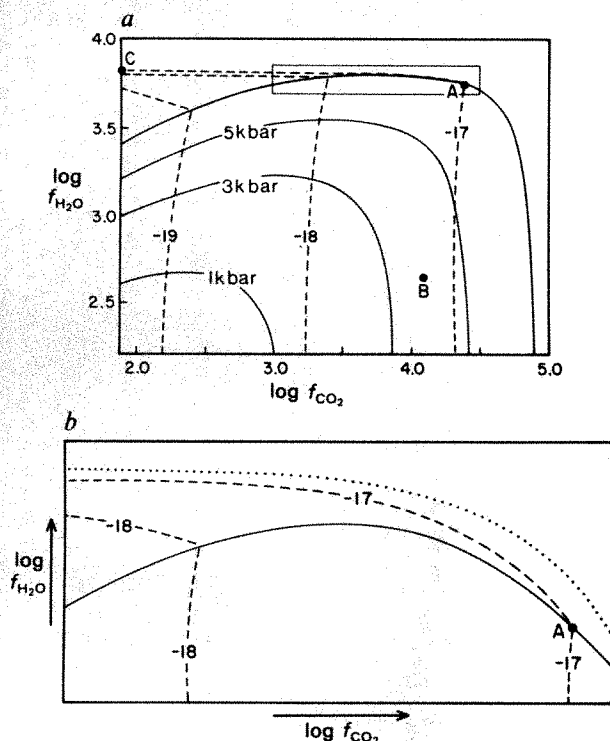


Fig. 1 *a*, *b*, C-O-H fluid equilibria at 700°C, $P_L = 7$ kbar. Graphite is stable only along the outermost solid curve. Outside this curve $\alpha_C < 1$ at $P_F = 7$ kbar. Inside this curve $P_F < 7$ kbar so that a freely-migrating fluid at lithostatic pressure cannot exist within this region. Isopleths of $\log f_{O_2}$ (dashed) show the effect of an oxygen buffering mineral assemblage (these isopleths are calculated assuming $\alpha_C = 1$ within the graphite stability curve). *b*, An enlargement of the area within the box in *a*, showing the intersection of the $\log f_{O_2} = -17$ isopleth with the graphite stability curve at point A. At this point $X_{CO_2} \approx 0.3$ and addition of CO_2 will precipitate graphite. For these conditions the amount of CO_2 necessary to stabilize 10 vol. % orthopyroxene would precipitate 1.5 vol. % graphite.

five remaining variables. In a graphite-bearing system where $\alpha_C = 1$, it is thus possible to calculate the fugacity of five fluid species by determining the fugacity of one. If graphite is not present the determination of the fugacity of two fluid species allows the determination of the other four as well as α_C .

The calculations summarized here were performed by the program CALCOH which employs a hard-sphere modified Redlich-Kwong equation of state for H_2O , CO_2 and CH_4 (refs 12, 13), and the Gibbs energy of each fluid¹⁴. Calculations of fluid fugacities were made with and without consideration of non-ideal mixing by these three fluid species, with a general agreement of better than 0.1 log unit.

Results of these calculations are shown as a function of $\log f_{CO_2}$ versus $\log f_{H_2O}$ at constant $T = 700^\circ C$, $P_L = 7$ kbar (Fig. 1*a*, *b*). The outermost solid curve delineates the stability field of graphite. Along this curve $\alpha_C = 1$, $P_F = P_L = 7$ kbar, and knowledge of the fugacity of any one of the six fluid species determines the system. Any point inside (below and to the left of) the graphite stability curve is equivalently fixed for a lower P_F as indicated by the 1-, 3- and 5-kbar lines. These pressure contours are calculated assuming $\alpha_C = 1$. It is important to point out that for a graphite-absent rock, where $\alpha_C < 1$, these contours become the upper limit to P_F . Thus, at $P_L = 7$ kbar there will not be a free-fluid phase for values buffered inside of the graphite stability curve as $P_F < P_L$. The dotted curve (Fig. 1*b*) satisfies the relation $P_{H_2O} + P_{CO_2} = 7$ kbar. As high-temperature rocks are ductile with relatively little strength, the region above this curve is geologically inaccessible as a fluid overpressure would result, allowing fluids to escape. The $\log f_{O_2}$ contours are calculated at $P_F = 7$ kbar outside (above) the graphite stability curve at $\alpha_C < 1$, and $P_F < 7$ kbar inside graphite stability at $\alpha_C = 1$. Only

on the graphite stability curve can $P_F = 7$ kbar in the presence of graphite ($\alpha_C = 1$). An important ramification of this diagram is the instability of 7 kbar of fluid inside the graphite stability curve.

The significance of this result is seen for a rock where $\log f_{O_2}$ is buffered to -17 at 700°C, 7 kbar (dashed isopleth in Fig. 1). At low f_{CO_2} (near point C) graphite is unstable at $P_F = 7$ kbar. However, if CO_2 infiltrates the rock it becomes the dominant fluid species at a very low CO_2/R as pore space will be low at these P and T and no other fluid species are assumed to be buffered (CO_2/R is the CO_2 /rock ratio calculated from the moles of oxygen; some workers prefer to use this weight ratio, the conversion being 1.0 atom ratio ≈ 0.5 wt ratio). If infiltrating CO_2 dilutes intergranular H_2O then the system will increase in f_{CO_2} (and α_C) following the $\log f_{O_2} = -17$ isopleth towards the right from point C and intersecting the graphite stability curve at point A at which time graphite becomes stable and $X_{CO_2} \approx 0.3$. The system is then invariant in the presence of graphite. Any additional CO_2 infiltration will be buffered and will drive reaction (1) to the left, precipitating graphite and exhausting the oxygen buffering capacity of the rock. As long as this buffering capacity is maintained CO_2 will be converted quantitatively to graphite and CO_2/R for infiltration can be calculated from the amount of graphite that is precipitated. Such CO_2/R values are considered maxima because some graphite might predate and be unrelated to CO_2 infiltration.

An estimate of the amount of CO_2 -flooding necessary to form a granulite can be made from modal analysis and consideration of reaction progress for dehydration reactions. For example, based on the idealized stoichiometry of the amphibole breakdown reaction:



it is estimated that in certain rocks from the Adirondack Mountains¹⁵ H_2O was buffered at $X_{H_2O} = 0.12$, at 700°C and 7 kbar. If 10 vol. % orthopyroxene formed by reaction (6) in response to CO_2 infiltration then CO_2 must have continuously diluted all evolved H_2O . In this example, H_2O must always comprise 12 mol.% of the fluid ($X_{CO_2} \approx 0.88$), and the total CO_2/R is estimated at 0.1. If conditions of $X_{H_2O} \approx 0.1$ from the Adirondacks^{8,15,16} are similar to those of other granulite terranes then values of $CO_2/R \sim 0.1$ should generally be necessary to form granulites by CO_2 flooding. This value is, however, a conservative estimate and values of $CO_2/R = 0.25$ have been independently estimated². Higher CO_2/R values would cause precipitation of larger amounts of graphite in reduced conditions.

In a rock with < 0.1 vol. % pore space, infiltration equal to $CO_2/R = 0.1$ will precipitate ~ 1.5 vol. % graphite. At low porosity, the amount of graphite precipitated depends only on CO_2/R and is independent of f_{O_2} as long as f_{O_2} is buffered to within the stability of graphite. If a high pore volume of 1% is chosen then the amount of graphite is negligibly reduced to 1.47%. Regardless of its form, as little as 0.1% graphite is easily identified either macroscopically or in thin section allowing reliable identification of rocks that have undergone very small amounts of infiltration ($CO_2/R > 0.007$). Thus it would be difficult to miss evidence for CO_2 infiltration in low f_{O_2} rocks.

The general applicability of these calculations is dependent on what values of f_{O_2} are buffered during granulite facies metamorphism. If f_{O_2} is buffered below reaction (1) then large amounts of readily observable graphite must be present to satisfy models of CO_2 flooding, while if f_{O_2} values exceed the stability of graphite then the absence of graphite provides no information as to CO_2/R .

Unfortunately, the presence of graphite by itself does not necessarily indicate CO_2 infiltration even though it obviously signifies f_{O_2} values less than reaction (1). The problem arises because much graphite of premetamorphic, biogenic origin is preserved in granulites¹⁷, and thus the presence of graphite, which may be 2–10 vol. % in some deposits¹⁸, indicates CO_2 infiltration only if lower grade equivalents are graphite-free. Glassley³ concludes that CO_2 flooding is indicated by the

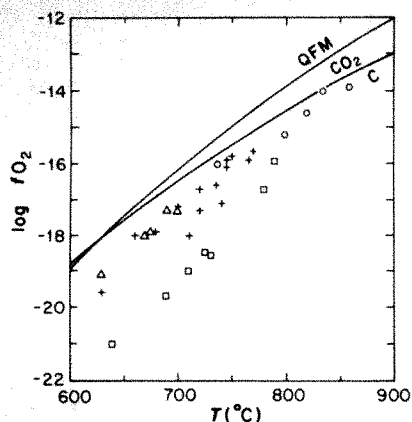


Fig. 2 Values of f_{O_2} for granulite facies samples estimated from coexisting magnetite + ilmenite. Reaction (1), which closely approximates the maximum f_{O_2} stability of graphite, and QFM²⁶, the quartz + magnetite + fayalite O_2 -buffer, are shown for reference at 7 kbar. Samples: $\circ$, from Scourie²⁵; $\square$, from Quebec²⁴; $\triangle$, from south Quebec²³; +, from the Adirondacks²². Graphite was not reported in most samples indicating that if CO_2 infiltration occurred, amounts of CO_2 were very small, $CO_2/R < 0.007$, and were not sufficient to account for granulite formation.

presence of graphite in two granulite terranes, but this has been criticized on the grounds that graphite-absent amphibolite facies rocks are not correlative with graphite-bearing granulites¹⁹. The great difficulty in correlating across the amphibolite-granulite transition has thus far prevented successful application of this test; however, the presence of small amounts of graphite is still restrictive as it places an upper limit to CO_2 infiltration. For example, it has been estimated that 35% of the rocks in an area of the Marranguit Peninsula, West Greenland contain graphite at levels of $< 0.01\%$ (ref. 20). If all of this graphite was precipitated by CO_2 infiltration then a CO_2/R value of < 0.0007 is indicated, which is not enough to form these granulites. If some graphite is biogenic in origin then CO_2/R would be less, possibly zero.

Coexisting Fe-Ti oxides have been successfully used to estimate f_{O_2} and temperature in a wide range of igneous and high-grade metamorphic terranes²¹. Values from four studies of granulites are shown in Fig. 2 (Adirondacks²², Quebec^{23,24}, and Scourie²⁵). These values are from several rock types including charnockites, anorthosites, metagabbros, granites, trondhjemites and a titaniferous magnetite deposit. Because most f_{O_2} values plot below reaction (1), even when considering an uncertainty of $\log f_{O_2} \pm 0.5$, it is clear that most of these samples should contain graphite if they were infiltrated by CO_2 . No graphite is reported for any of the samples from refs 22, 23 and 25. We have further examined the Adirondack samples²², both in thin section and macroscopically, but have observed no graphite at the 0.01% level.

The assemblage, biotite + K-feldspar + magnetite + ilmenite, buffered f_{O_2} and f_{H_2O} in an Adirondack charnockite. The estimated values are $\log f_{O_2} = -17.2 \pm 0.4$ and $\log f_{H_2O} = 2.65 \pm 0.65$ (ref. 16). These values are plotted in Fig. 1 (point B), showing that if $\alpha_C = 1$ then P_F must have been ~ 4 kbar for this sample (if $\alpha_C < 1$ then P_F was less). Thus, these estimates indicate that no free-fluid phase could have coexisted with this rock at the pressure and temperature estimated for granulite facies metamorphism, unless the metamorphic fluid deviated substantially from the C-O-H system. We consider the presence of such great amounts of other fluid species (such as H_2S , N_2 , Ar, HF, NaCl) to be unlikely on a regional basis. Common orthopyroxene-bearing assemblages in granulites indicate $\log f_{H_2O}$ is significantly below 3.5 ($X_{H_2O} \leq 0.1$), and such low f_{H_2O} is generally ascribed to all granulite terranes based on melting equilibria as well as dehydration reactions. If the rocks in Fig. 2 were buffered at low f_{H_2O} then none of these samples could have been infiltrated by significant CO_2 as a free C-O-H fluid at $P_F = 7$ kbar would

not be stable. It is thus likely that all of these rocks represent fluid-absent granulite facies metamorphism.

Further studies are necessary before it is possible to generalize about the fluid conditions of the lower crust. These results show that some common, granulite facies rocks have not been flooded by sufficient CO_2 to explain their genesis. However, compelling evidence indicates that granulites from certain quarries in southern India were stabilized by infiltrating CO_2 (refs 2, 4, 6). Graphite is not reported in these rocks, but since f_{O_2} is buffered to above QFM by the reaction 3 ferrosilite + $1/2 O_2 =$ magnetite + 3 quartz (R. Newton, personal communication), graphite is not expected regardless of whether CO_2 flooding occurred or not. Thus the results reported here may not be at variance with those from southern India. Extreme gradients in f_{CO_2} and the absence of pervasive fluid movement is reported for some localities in the Adirondacks⁸. If such heterogeneous fluid conditions are representative of granulite facies terranes elsewhere, then low f_{H_2O} values may indicate either extraction of magmas, channelized CO_2 infiltration, or metamorphism of already dry rock, and these processes may operate in close proximity. Very real contrasts in process may also exist from terrane to terrane due to differences in age and/or tectonic style.

This study was supported by grants from the Gas Research Institute (5083-260-0852), the NSF (EAR81-21214 and 83-11772) and the Geological Society of America Penrose Fund (3256-83). We thank P. E. Brown, T. C. Juster, E. J. Essene and W. E. Glassley for helpful conversations and reviews.

Note added in proof: We have used the calibration of Buddington and Lindsley²¹ to estimate f_{O_2} from Fe-Ti oxides rather than that of Spencer and Lindsley²⁷ (1981) (D. H. Lindsley, personal communication). More recently, unpublished experiments of Anderson and Lindsley yield somewhat lower T at low f_{O_2} (D. H. Lindsley, personal communication) and suggest that the oxides from Quebec²⁴ have reset after metamorphism. Most of the other values in Fig. 2 are not significantly affected and thus the conclusions of the study are not changed.

Received 11 May; accepted 24 July 1984.

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Black acidic snow in the remote Scottish Highlands

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Parts of Scotland experience large acidic depositions^{1,2} which may result in ecological damage³. Some highland areas appear to suffer enhanced acidic input² which frequently exhibits pronounced episodicity⁴. Acidic snowfall leads to acidic flushes during snow-

pack melting^{5,6}. Polluted grey snow layers have been reported in Scandinavia⁷⁻¹⁰, but we present the first documented case remote from major pollution sources of a distinctive black, acid snowfall episode with pH 3.0. The particulate deposit was very large and consisted of ~29% carbon; the remaining fraction included particles which could be identified as coal fly-ash. The heavily polluted air originated to the south-south east and was probably transported at an altitude of ~1,000–1,500 m in association with a stable atmospheric layer whereupon particles were efficiently scavenged by snow. Such transport and deposition mechanisms may produce the greater pollutant deposition sometimes observed in mountain areas². Similar events may not be rare and could make an important contribution to the annual pollutant input.

Shortly before 06.00 on 20 February 1984 a black snowfall (~5 cm) fell in the Cairngorm mountains (data from Aviemore Meteorological Station). Our survey, by road and foot transects in the more accessible zones, indicated that ~200 km² were affected. Individual samples (22) were collected within hours of falling. Because of the difficult conditions, the sample locations were not equally spaced but were biased towards those that appeared to be close to the eastern and western extremities of the fall. The particulate loading was exceptionally heavy (~0.01 g l⁻¹ of melted snow) and our conservative estimate is a deposit of 0.1 tonne km⁻². Detailed analysis has been undertaken on samples collected at 1,080 m, OS National Grid NJ013046. Our survey of snow packs southwards to Edinburgh, a few weeks later, revealed no trace of the event which by then had evolved into a distinctive grey layer ~40 cm thick in the Cairngorms. Local residents claim that black snow has fallen a number of times annually in recent years and appears to favour higher altitudes.

Figure 1a shows the synoptic pattern. For several days preceding the event, a ridge from a strong anticyclone to the east influenced the eastern side of the United Kingdom. Pronounced anticyclonic subsidence inversions were observed (Fig. 2) and at 00.00 on 20 February 1984 the lapse rate in the sub-inversion air was close to the dry adiabatic value, unlike the previous day when it was relatively stable¹². Snow showers, hail showers and cumulonimbus development were also observed in NE Scotland and off the coast at around 06.00 and during the following daylight hours¹². The lower boundary of the black snow layer was a thin stratum of hailstones.

Back trajectories for air over the Cairngorms are shown in Fig. 1b. The air flowed over a broad corridor of industrial and urban activity from the Trent Valley and southern Yorkshire, northwards over Edinburgh and Dundee. Given the uncertainties of trajectory analysis, the large industrial area of Tyneside-Teesside could have contributed, as could smaller zones of activity. European sources could also have contributed to the overall atmospheric pollutant loading, as black atmospheric

Table 1 Composition of black snow and composition range of 12 other snows collected within 2–3 hours of falling during January–April 1984

	pH	SO ₄ ²⁻	Cl ⁻	NO ₃ ⁻	NH ₄ ⁺	Ca ²⁺	Mg ²⁺	Na ⁺	K ⁺	Charge balance
Black snow	3.0	19.8	14.8	20.9	0.3	0.63	0.52	4.52	0.39	+5.4%
Other snows	3.5–4.7	0.27–4.86	0.15–10.23	0.11–3.52	ND	ND	0.02–0.64	0.07–4.45	ND	ND

Ion concentrations are given in p.p.m.; pH was determined immediately on melting and after filtering through a 0.45-μm Oxoid membrane. Ion determinations were made after storage at 4 °C. Anions were determined on a Dionex Model 12 ion chromatograph (precision ~5%). Cations were measured on a Pye Unicam spectrophotometer (Na⁺, ±0.1; K⁺, Mg²⁺, Ca²⁺, ±0.02) except for NH₄⁺ which was determined by the indophenol blue method on an autoanalyser. There is some evidence that the charge balance is due to organic ions, although pH measurement errors may make a contribution. Charge balance is excess equiv./total equiv. ND, not determined.

particle episodes have been observed in Sweden with winds in the England to Poland sector¹⁹. Pollutant plumes from individual large sources can be tracked for hundreds of kilometres^{20–22} and when they become entrained within or beneath an elevated stable layer there may be little cross-wind dispersion and retention of plume coherence for considerable distances²⁰. The importance of transport above the mixing layer is that the process of dry deposition is inhibited and there is potential for very long range transport²³. Long-range transport of power plant plumes has been found, in the United States, to be favoured at heights of ~800–1,500 m at night when wind speeds were up to ~14 m s⁻¹ at this level; when surface wind speeds were <4 m s⁻¹; when emission was into the lower atmosphere with a dry adiabatic lapse rate and the plume became confined in a more (and becoming more) stable atmosphere²⁴. These atmospheric conditions show strong similarities with those prevailing in this case¹². Such stable plumes have been reported to be dry-deposited on high terrain at night²⁵.

The elevation of the mountains, the geography to the south, black snow distribution and the requirement for little-dispersed, heavily-polluted air to be available for scavenging suggest that advection of the greater proportion of the pollution was within or just below the subsidence inversion layer. This implies that the pollution would have to rise to about 1,000 m and implicates large emitters producing buoyant plumes, although low-level emissions may have made a contribution (synoptic observations of smoke and haze indicated a northward transport of pollution in the lower layers; Fig. 2). Power station plumes, for example, can rise to these heights under the light wind conditions²⁶ which prevailed during the previous night¹¹. Figure 2 shows the heavily polluted air being made available for snow scavenging over the Cairngorms. It was clear that snow scavenging, and not dry deposition, was the removal process as the particles were

Fig. 1 a, Synoptic pattern at 06.00 on 20 February 1984¹¹ with locations referred to in Fig. 2 (L, Lerwick; S, Shanwell; H, Hemsby). b, Back-trajectories from the Cairngorms at 00.00 (●) and 06.00 (—) on 20 February 1984, at 6-hour intervals. The geostrophic approximation for the surface pressure distribution was used. The plan of the 00.00 trajectory agrees well with the observed 850 mbar winds¹² although use of these would increase the length of the 6-hour trajectory by 40%. There are considerable uncertainties and debate over trajectory analysis^{4,13}. The 850 mbar wind has been used¹⁴ and the use of the surface pressure field is considered to be robustly appropriate^{15–18}. In this case the cross-isobar component of flow was restricted to a very shallow layer of air near the ground¹². Both trajectories are sufficiently similar for us to be confident (in the absence of an exact time for the event) of the likely pollutant source regions.

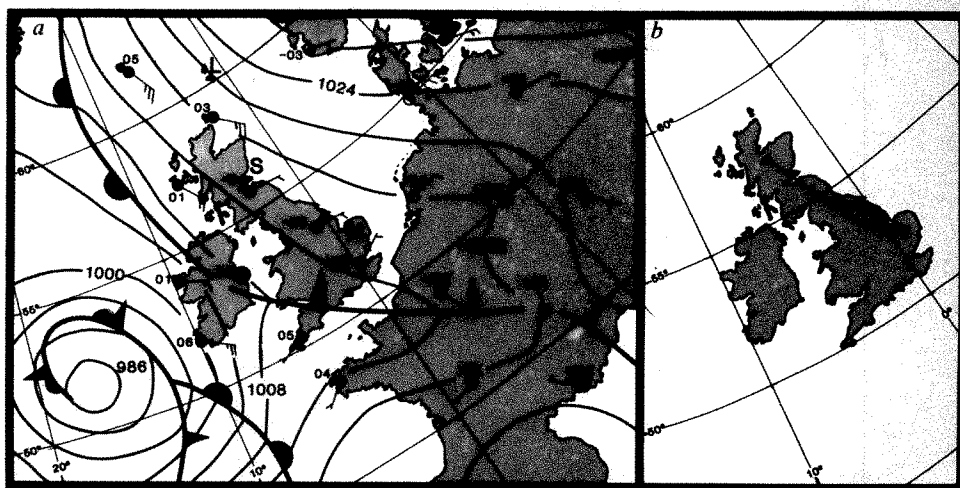
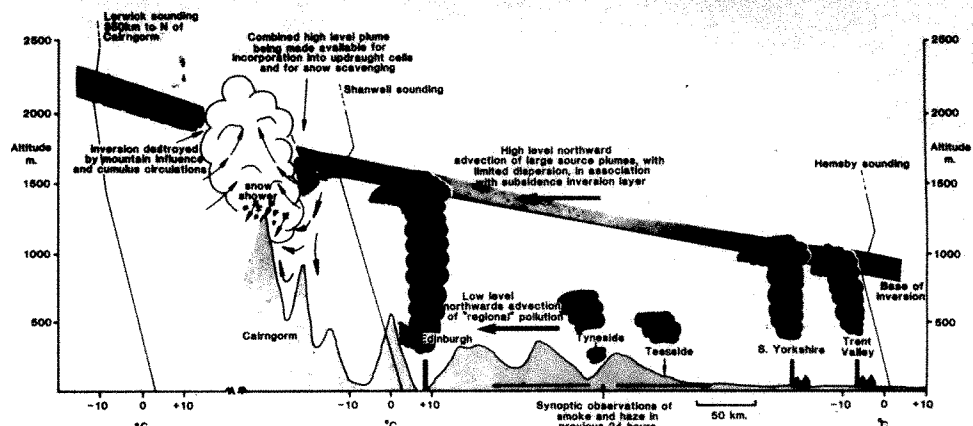


Fig. 2 Schematic cross-section along back-trajectory to the Trent Valley. Shown are dry-bulb temperature soundings at 00.00 on 20 February 1984 (see Fig. 1). Tyneside and Tees-side are industrial areas to the east of the probable trajectory, but may have contributed to the 'regional' pollution plume. Advection in association with the inversion layer made available little-dispersed, heavily-polluted air for scavenging. Pollution in the less stable, sub-inversion layer was more efficiently dispersed and diluted and further depleted by dry deposition, especially in hilly terrain. The strength and position of the inversion fluctuated temporally and spatially¹²; consequently the northward advection of the inversion-layer pollution should not be regarded as increasing in height in the simple fashion portrayed. No showers occurred along the trajectory south of the Scottish mountains¹¹ and so high pollutant concentrations in the mountain air were available for scavenging via down-mixing induced by topographic disturbance, and compensating downdraughts associated with the cumulus circulations. These cumulus circulations may have drawn in volumes of the heavily polluted air, effectively 'concentrating' the pollution to produce a pronounced localized deposition²⁶.



incorporated throughout the whole depth of the fall. Snow is a much more efficient scavenger of aerosols than rainfall^{27,28} and scavenging efficiency increasing with decreasing ice-crystal diameter²⁹. This episode consisted of fine-grained snow.

The black snow had a pH of 3.0 at the main sampling site. The volume-weighted mean annual precipitation in this area of Scotland is around 4.5 (ref. 2), although the pH is lower for our recent snow samples from greater elevations (Table 1). Of particular interest are the relatively large excess of Cl^- and the NO_3^- concentrations in the black snow. HCl is very soluble and so will be relatively easily removed once precipitation occurs. NO_x oxidizes before SO_2 (ref. 30) and snow is relatively efficient at removing NO_3^- (refs 28, 29). The molar ratios of NO_3^- : non-sea salt SO_4^{2-} : non-sea salt Cl^- (1.71:1.00:1.04) may therefore reflect pollution plume ageing, different solubilities and differential scavenging, or different sources.

Fog or cloud droplet deposition may increase acidic input at this altitude³¹, but our observations appear to show a systematic increase of pH with height (Table 2), suggesting that this was not the most important mechanism in this case. Snowflakes at lower elevations had a longer fall-path through polluted air mixed down into the mountains from high level (Fig. 2) or, if the cloud enveloped the mountains, the mechanism may have been early scavenging of ascending polluted air drawn into the cloud.

Figure 3 shows many particles which are typical of pulverized fuel (PF) or coal fly ash^{32,33}. The carbonaceous component, which gave the snowfall its distinctive appearance, comprises much of the particulate volume but it is highly porous (with a specific area of up to 80 times that of non-carbonaceous oil-fired fly ash³⁴). The carbon content of the particulate is $29 \pm 1.5\%$ by weight (Hewlett-Packard 185 B CHN analyser). The carbon content of chimney solids from modern UK oil-fired power stations is $<5\%$ by weight³⁴, but values in excess of 20% have been reported in the United States under some operating conditions^{32,35}. Other coal burners produce larger proportions of carbon residue and would have contributed to the black snow carbonaceous component. For example, the carbon content of domestic coal ash is very high³⁴. The carbonaceous residue particles in PF fly ash are often concentrated in the fraction greater than $45 \mu\text{m}$ in diameter³⁴, and as they are highly porous, they will become relatively enriched at the expense of other, heavier fly ash components which settle out much more rapidly in the long-range transport of the plume. In addition, the large surface area of the carbonaceous particles will lead to further enrichment in the snow scavenging process. The carbonaceous residue may be of particular interest to the acidic deposition issue as it has a large capacity for absorbing acids in the plume. The pH of a soot/water slurry decreases with time whereas that of a non-carbonaceous PF fly ash/water slurry increases³⁴.

Besides glassy spheres and the very porous carbon back-ground, other less easily identifiable particles are seen in Fig. 3 and these may be from a variety of sources. Elemental proportions (Table 3) are similar to those of PF fly ash^{35,36} (and perhaps to other industrial ashes though data are not available) although Ca proportions are relatively low (the Ca fraction is soluble, and there are high concentrations in the meltwater) and Fe is higher than 'typical' but well below the 'common maximum'³⁷. The Pb, Mn and Zn concentrations (respectively $1,530 \pm 11$, $4,960 \pm 94$, $9,830 \pm 108$ p.p.m. after HNO_3 and HClO_4 digestion and atomic absorption spectrophotometry) are higher than those typically observed in PF chimney solids³⁸ (especially Zn) but trace elements, in particular Zn³⁹, are known to be enriched on small particles⁴⁰⁻⁴³ which have much longer atmospheric residence times. The log probability size distribution (Malvern 3600 E-type particle size VA3 analysis after ultrasonic dispersion) for the fraction $<4 \mu\text{m}$ diameter ($\sim 70\%$ of the total number) was similar to distributions determined for PF chimney solids⁴⁰, but shifted towards smaller diameters. Our distribution was deficient in $>4 \mu\text{m}$ particles, presumably as a result of preferential removal in the ageing atmospheric plume (this is not inconsistent with carbonaceous component enrichment in the plume when the weight of carbon as a proportion of different size classes of fly ash from PF power stations is considered³⁴).

In the region of the back trajectory there is a total of $\sim 20,000$ MW of PF power station capacity, the largest plant in

Table 2 Snowfall pH variation with altitude on a hill 18 km to the west of Cairngorm

Altitude (m)	280	295	320	410	500	570
pH	2.95	3.00	3.10	3.15	3.30	3.30

It is possible that this variation reflects an 'edge' effect as this was the most westerly sampling point and altitude increased towards the west.

Table 3 Range of percentage contribution of measured elements to the total weight of all 14 constituent elements in black snow samples

Na	2.2-5.4	Ca	0.6-1.1
Mg	4.0-7.5	Ti	0.5-1.3
Al	18.6-24.0	V	0.0-0.1
Si	30.3-44.3	Cr	tr-0.1
S	1.3-1.8	Fe	18.9-21.4
Cl	0.3-0.4	Ni	0.1-0.15
K	1.9-5.1	Cu	0.05

Values obtained by EDAX bulk analysis⁴⁰ over areas of 20 mm^2 of membrane filters (three analyses). The accuracy of determination for each element is around $\pm 5\%$. tr = trace.

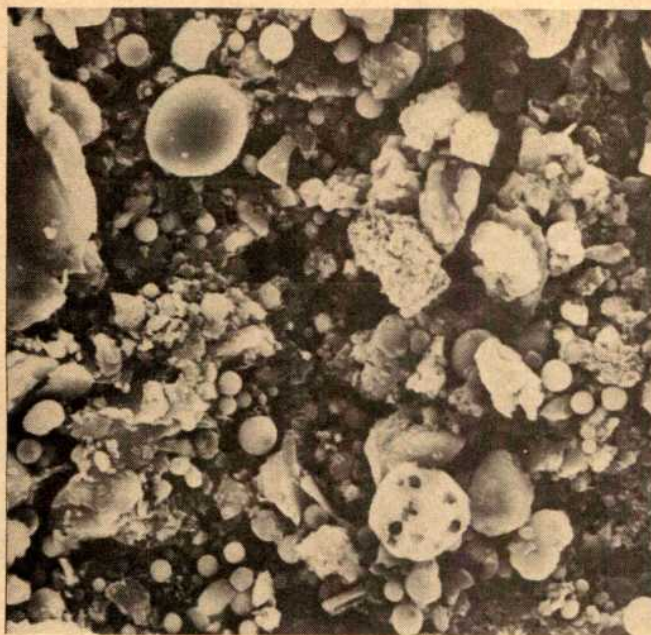


Fig. 3 Scanning electron microscope photograph of particulate material which shows many glassy spheres generally $<5\ \mu\text{m}$ diameter. The spongy, lattice-like background is carbon residue. The large sphere to top left is $\sim 8\ \mu\text{m}$ diameter. The cenosphere (porous particle near bottom) may be tarry, coal exudate but is a typical product of oil-firing (they are relatively infrequent in our samples, ~ 1 in 200).

the Trent Valley and southern Yorkshire. Other large buoyant sources include chemical plant near Teesside and an oil refinery (with its own 100-MW oil-fired power station) to the west of Edinburgh (although our trajectory analysis favours a route to the east of the city). Areal sources in cities may have also been sufficiently buoyant to become entrained in the high-level advection. No information is available on the nature of emissions from the refinery although large quantities of SO_2 , NO_x and organics are released from plant in the United States⁴⁴. The refinery particulate discharge is likely to be small⁴⁴ compared to the 20 tonnes per day suggested for a modern UK 1,800-MW PF power station (generating at full load and assuming an ash capture efficiency of 99.3%)⁵⁴. It is expected that oil ash would contain much higher V and Ni concentrations than our samples (166 ± 50 , 176 ± 10 p.p.m. respectively) and more cenospheres⁴⁵.

Whatever the major source(s) of this large pollutant deposit it is likely that disparate activities have contributed. The exposed faces of the evolved grey snow layer produced a distinctive odour which pervaded the mountainside under calm conditions. It was difficult to characterize the smell and to identify the agents, but some observers have described it as an acetone/phenol-type odour. Petrochemical sources may have contributed. The heavy particulate deposit was the most remarkable feature of this episode. For example, it would require the entire emission for more than two hours from $\sim 20,000$ MW of efficiently-operating PF power stations³⁴.

Such severe acidic episodes could enhance the spring snow pack 'acid flush', both through heavy loading and the 'banding' of acidity in the snowpack (A. R. W. Marsh and A. H. Webb, unpublished CEBG report, 1978). It is of interest to note that the weather pattern was similar to that which produces acidic episodes in Scandinavia⁴⁶, from widespread sources. There is some evidence that the frequency distribution of this type is changing on time scales from years to decades (T. D. Davies and P. M. Kelly, unpublished work) and there may be implications for dry as well as wet deposition in polluted air volumes retaining coherence over long distances and becoming available for transfer to the ground in mountain areas²⁵.

We are grateful for the financial support of the NERC and EEC. We thank Dr A. G. Thomas, Institute of Hydrology, for

assistance with NH_4^+ determinations, and the Central Electricity Generating Board for helpful comment and discussion.

Received 18 June; accepted 12 September 1984.

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Cell size control of development in *Saccharomyces cerevisiae*

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Diploid cells of the yeast *Saccharomyces cerevisiae* in the G_1 phase of the cell cycle are faced with the alternatives of either continuing vegetative cell division or undergoing the developmental processes of meiosis and subsequent ascospore formation, or adapting to starvation conditions if these apply. The course taken is influenced by the nutritional status of the culture medium¹, the presence of both *MATa* and *MAT α* mating-type alleles², and the need for cells to be in the G_1 phase of the cell cycle³. For those cells that continue cell division, size controls operate in both the budding yeast *S. cerevisiae*^{4,5} and the fission yeast *Schizosaccharomyces pombe*^{6,7}. In *S. cerevisiae* the 'start' event initiating the cell cycle is controlled in some way related to cell size because cells below a critical size fail to initiate cell division⁸. The ability of cells to undergo the developmental process of sporulation is related to cell age, in that cells gain this ability just before the emergence of the first bud⁹ and the process of sporulation after initiation is altered in small cells¹⁰. Here we report that the initiation of sporulation is subject to a size control related to absolute cell volume, which is distinct from control by cell age and also independent of the control operating on the initiation of cell division.

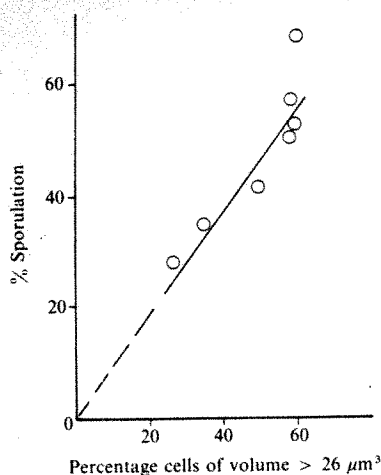


Fig. 1 Relationship between the ability of a yeast population to sporulate and the percentage of cells of volume $>26 \mu\text{m}^3$ before the shift to sporulation medium. Cells of strain S41 grown aerobically in continuous culture at dilution rates between 0.02 h^{-1} and 0.09 h^{-1} were examined microscopically and individual cell volumes measured. Cells were collected from 30 ml samples, washed and resuspended in sporulation medium (20 g l^{-1} potassium acetate). After 48 h incubation at 30°C with shaking, the sporulation frequency of the samples was measured. Further samples were taken after 72 h to check that sporulation was complete. The chemostat medium contained (per l): 10 g galactose, 5 g ammonium sulphate, 1.5 g Difco yeast nitrogen base, 1 g Oxoid yeast extract, 0.1 g arginine, 5.5 g potassium dihydrogen phosphate, 10 g dipotassium hydrogen phosphate and amino acids at 0.1 g, pH 7.2.

Initially, it was noted in strains carrying the *whi1* or *whi2* size control mutation^{11,12} alone or in combination with the *spd1* mutation which derepresses sporulation¹³, that smaller cells in the population did not sporulate whereas the few larger cells present did¹⁴. These preliminary experiments suggested that *whi1* homothallic diploids were unable to sporulate, but did not indicate whether this was a direct effect of the *whi1* mutation, or simply an effect of the smaller cell size. This was tested by growing cells in a presporulation medium containing hydroxyurea, an inhibitor of DNA synthesis which arrests nuclear division without preventing mass growth, and therefore causes cells to become enlarged¹⁵. The results are shown in Table 1: *whi1* cells which were normally unable to sporulate could form large numbers of asci when made larger by preincubation with hydroxyurea. This indicates that the *whi1* mutation was not directly preventing sporulation, but was affecting the process through its property of reducing cell size. These results, while indicating that there may be some size control over initiation of sporulation, are subject to other interpretations as mutants were used.

By growing wild-type yeast at slow growth rates in continuous culture, very small cells can be obtained, and cultures with widely varying average cell sizes can be produced by changing the dilution rate¹⁶. We have studied the dependence of sporulation on cell size by growing populations in chemostat cultures over a wide range of dilution rates. Cultures were grown in media in which sporulation is not initiated but in which mitochondrial functions that are essential for spore formation are derepressed. At each steady state the distribution of cell sizes in the cultures was estimated microscopically and samples of the culture were transferred to sporulation medium to assess the extent to which that population of cells was competent to sporulate. From the results, we estimated the average cell size at which half the maximum sporulation frequency was obtained (that is, 40%); this gave an estimate of the critical volume for initiation of sporulation of $26 \mu\text{m}^3$. Figure 1 illustrates the linear relationship between the extent to which a culture sporulated on resuspension in sporulation medium and the proportion of cells of volume $>26 \mu\text{m}^3$ before the shift to sporulation medium.

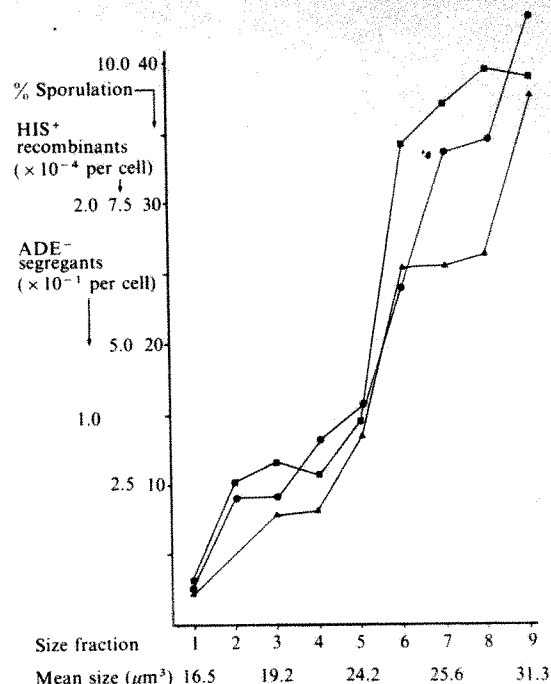


Fig. 2 Cells of strain 60x61,

<i>MATα</i>	<i>HO</i>	<i>HMLα</i>	<i>HMRα</i>	<i>ade2-40</i>	<i>his4-239</i>	<i>can1</i>	<i>CYH2</i>	<i>ARG4</i>
<i>MATα</i>	<i>HO</i>	<i>HMLα</i>	<i>HMRα</i>	<i>ade2-119</i>	<i>his4-166</i>	<i>CAN1</i>	<i>cyh2</i>	<i>arg4-1</i>

were grown in the chemostat medium described in Fig. 1 legend. This strain requires histidine for growth but not adenine. On meiotic recombination, however, it yields histidine prototrophs and on chromosome segregation it yields red adenine auxotrophs. Cells were collected from 10 ml of chemostat culture, washed in sporulation medium, and resuspended in the least possible volume. The suspension was loaded onto a gradient of Urografin (density $1.04\text{--}1.08 \text{ g cm}^{-3}$, made up in 2% potassium acetate) in 16.5-ml tubes, and centrifuged for 13 min at 60 g. After centrifugation, the part of the gradient containing the cells was split into nine fractions and the cells from each fraction were collected and resuspended in 3 ml of sporulation medium. Samples of each fraction were diluted appropriately and plated onto minimal medium (1.5 g l^{-1} Difco yeast nitrogen base, 5 g l^{-1} $(\text{NH}_4)_2\text{SO}_4$, 20 g l^{-1} glucose, 20 g l^{-1} agar) and YEPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose, 20 g l^{-1} agar) immediately after resuspension and after 48 h incubation at 30°C . The figure shows the percentage sporulation (●), appearance of histidine prototrophs (▲), and appearance of adenine red auxotrophs (■) in each size fraction.

These data could be interpreted in terms other than the existence of a critical cell size control over initiation of sporulation as: first, they only refer to the production of mature ascospores and therefore cells may be initiating, but not completing sporulation; and second, they only deal with average cell sizes of populations, and not with individual cells. The first of these possibilities was excluded by examining the frequency of intragenic recombination at the *HIS4* locus in populations of sporulating cells of different sizes obtained from a single parent culture by density gradient fractionation. The results (Fig. 2) show that small cells were not undergoing meiotic levels of intragenic recombination at the *HIS4* locus or chromosome segregation. Thus, small cells do not proceed as far as the early sporulation event of intragenic recombination.

The sporulation of individual cells was examined by growing cells of strain 60x61, heterozygous at the *ADE2* locus, in a medium giving a range of cell sizes, resuspending them in sporulation medium, and after 20 h of incubation, dissecting out 250 cells of $<30 \mu\text{m}^3$ volume, and 256 cells of $>40 \mu\text{m}^3$ volume. On subsequent incubation, 110 of the latter were found to form red or sectorial colonies, indicating segregation at the *ADE2* locus, while none of the former showed segregation. We were able to measure cell sizes after 20 h in sporulation medium

Table 1 Hydroxyurea restores the ability of *whi1* mutants to sporulate

	Mean cell volume (μm^3)	% Ascus formation
Wild type	78.2	65.4
Wild type (HU-treated)	ND*	62.3
<i>whi1</i>	22.1	1.6
<i>whi1</i> (HU-treated)	50.4	29.0

Cells of strains S41:	<i>MATa HO HMLa HMRa arg4-1 cyh1</i>
	<i>MATa HO HMLa HMRa arg4-1 cyh1</i>
and 65:	<i>MATa HO HMLa HMRa whi1 met5</i>
	<i>MATa HO HDLa HMRa whi1 met5</i>

were inoculated into YEPAG medium (10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone, 20 g l⁻¹ potassium acetate, 1 g l⁻¹ galactose), and after 2 h incubation, hydroxyurea HU; 50 mM) was added to half the samples and incubation was continued. When the absorbance at 600 nm reached 1.0, the cell size distribution of each culture was measured microscopically, and the cells were transferred to sporulation medium. After 48 h in sporulation medium, the frequency of asci in each culture was measured. ND, not determined.

* Large elongate cells of irregular shape.

as cell size in this strain did not significantly increase during sporulation.

The sporulation of individual cells was also examined by photographing 350 cells before and after sporulation on solid medium. No cells smaller than 25 μm^3 sporulated, and the probability of sporulation increased with increasing size above 25 μm^3 , up to a maximum frequency approaching 100% in cells larger than 130 μm^3 .

The cell size control over sporulation was distinguished from the cell age control by examining the sizes at which cells were able to initiate budding. Small cell fractions (obtained by methods described in Fig. 2 legend) were resuspended in rich growth medium and the sizes of budded cells were measured. The minimum size of budded cells was ~17 μm^3 , and about 8% of budded cells were <26 μm^3 in volume. This indicates that some cells of <26 μm^3 volume were capable of budding, therefore the inability of cells smaller than 26 μm^3 to sporulate was not simply due to their being unbudded daughter cells.

We have used a variety of different methods which give consistent results: yeast cells with a volume less than ~26 μm^3 are unable to sporulate, and this effect is unrelated to size control over budding and cell age control over sporulation. These results have interesting implications for other developmental systems. Size (or critical cell mass) control of cell division seems to operate in higher eukaryotes¹⁷ and similar effects may therefore influence the course of higher cell development.

This work was supported by grants from the SERC. We thank P. Sudbery, B. L. A. Carter and J. R. Dickinson for helpful discussion.

New evidence that growth in 3T3 cell cultures is a diffusion-limited process

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When a confluent culture of 3T3 cells is wounded, new growth occurs at the wound margins. This indicates that the suppression of growth in the intact confluent sheet is under local control, a phenomenon known as 'topoinhibition'¹, and it has been suggested that intercellular contact is responsible. An alternative explanation for topoinhibition is possible, however, namely that a soluble factor necessary for growth is locally depleted from the medium by cells so that each cell in a confluent sheet normally receives an insufficient supply and its growth is inhibited²⁻⁵. Here we show that the pattern of release from topoinhibition in a wounded culture can be distorted simply by inducing a gentle laminar flow of medium across the wound. Growth remains suppressed at the upstream margin of the wound despite the reduced level of intercellular contact at wound edges. We conclude that the signal for topoinhibition is carried by the flow as would be predicted if it were the local depletion of growth factor.

Hydrodynamic theory predicts the existence of a thin layer of non-turbulent medium next to the cells in which diffusion is the main mechanism of transport. Any growth factor consumed by the cells would thus become locally depleted in this boundary layer. This explanation of topoinhibition is reinforced by the discovery that forced turbulent stirring of the culture medium using either a miniature pump³ or vigorous shaking of the cultures⁴ increases the growth rate of 3T3 cells. Stirring reduces the thickness of the boundary layer and could, therefore, increase the diffusive flux of growth factor to the cells³⁻⁵; however, this interpretation has been criticized on the grounds that stirring could disrupt intercellular contacts and so the alternative hypothesis of growth control by contact is not eliminated⁶. No effects of fluid shear stress on intercellular contacts have been found although shear stress does affect the migration⁷ and cytoskeleton⁸ but not the growth⁷ of endothelial cells in culture. A further criticism is that increasing the viscosity of the medium does not have the effect of reducing the saturation density of 3T3 cultures that might be expected if growth depended on diffusive flux⁶. Thus, we have developed a direct test of whether the release from topoinhibition at wound margins depends on a diffusible factor.

3T3 cells were grown in Petriperm culture dishes in order to maintain steady conditions of pH and gaseous exchange. Confluent monolayers were wounded by scraping away a narrow strip of cells along a diameter of each dish and the medium was replaced with fresh medium containing ³H-thymidine and varying concentrations of serum. In some cultures, the lids were replaced with the apparatus shown in Fig. 1 and the cultures were all incubated for a further 16 h then prepared for

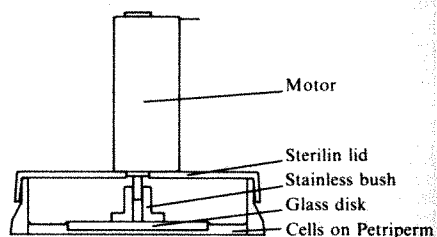


Fig. 1 Diagrammatic section of the apparatus. The motor (Portescap Ltd, type M915L, ratio 1:2,430), which is powered by a constant voltage source, is attached to a Sterilin lid which provides a better location on the Petriperm dish than a Petriperm lid. The 32-mm diameter glass disk is glued to the stainless steel bush and adjusted along the motor spindle to give a separation of 0.8 mm from the Petriperm membrane.

Received 22 June; accepted 21 August 1984.

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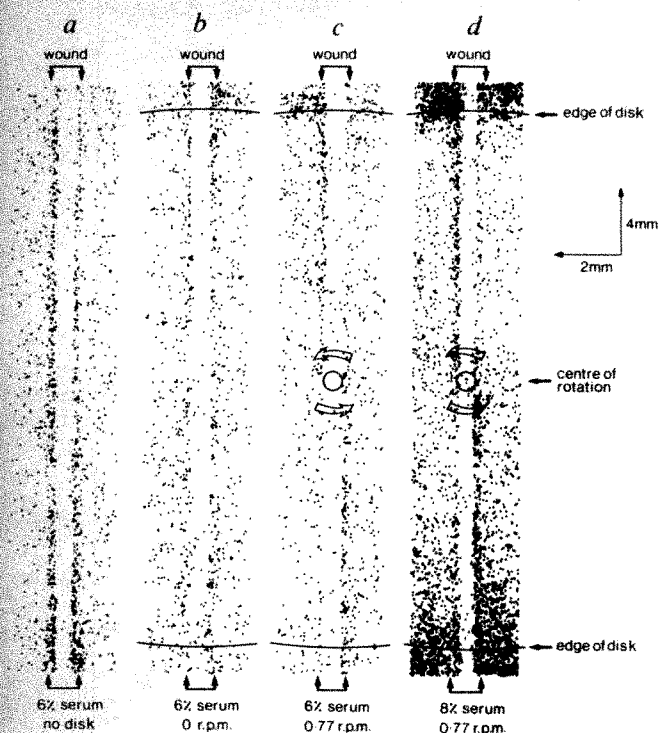


Fig. 2 Effect of different flow regimes and serum concentrations on the distribution of labelled nuclei at wound edges. Swiss 3T3K cells (obtained from Dr C. O'Neill) were grown in bicarbonate-buffered Dulbecco's modified Eagle's medium (Flow) containing 10% fetal calf serum (Seralab) in a 37°C humidified incubator gassed with 15% CO₂ in air. 3×10^5 cells were seeded in 5 ml of medium in 5 cm-diameter Petriperm dishes (Heraeus). After 60 h the medium in the cultures was replaced and on day 5 the cultures were used for the experiment, by which time they had been confluent for 2 days. Precise parallel-sided diametral wounds, 0.9 mm wide, were made with a stainless steel tool guided by a jig. The medium was replaced with 3 ml of fresh medium containing $1 \mu\text{Ci ml}^{-1}$ ³H-thymidine (Amersham International; specific activity 112 Ci mmol^{-1}). In *b*, *c* and *d* the original Petriperm lids were replaced with Sterilin lids bearing motors and the cultures returned to the incubator for 16 h. The cultures were fixed in ice-cold 5% trichloroacetic acid, rinsed in 95% ethanol and air-dried. Autoradiography was performed by attaching stripping film to the cultures, exposing them for 3 days, then developing and fixing. The grain density over labelled nuclei was so high that individual grains could not be distinguished and labelled nuclei could be identified using digital image analysis. A Zeiss RA microscope equipped with luminar objectives and a video camera was used to input a frame showing part of the wound to a digital image analysis system. The background was subtracted and windows for size, shape and grey level were set to identify labelled nuclei. The scale used for plotting nuclear positions is foreshortened along the axis parallel with the wound, as indicated by the two scale bars.

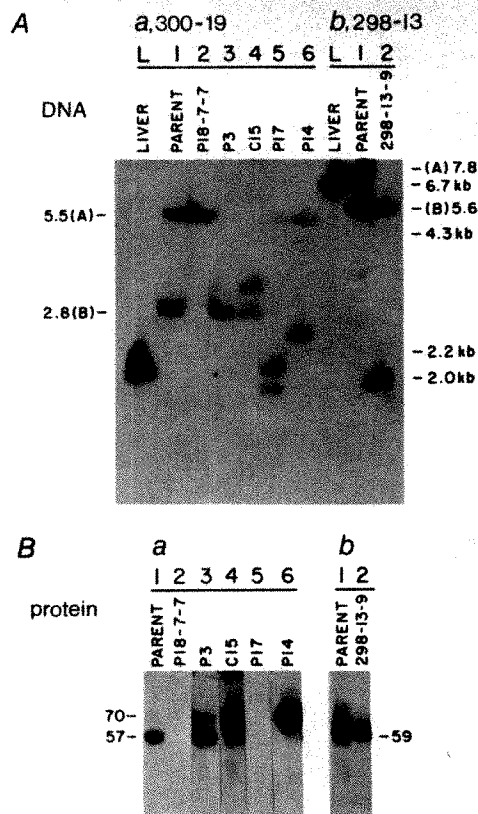


Fig. 3 *a*, Specimen fixed immediately after wounding and stained with Giemsa to show the original parallel-sided wound in a confluent sheet. *b*, Part of the same specimen as Fig. 2*d* but stained with Giemsa. Arrows show direction of flow; most labelled nuclei can be seen on the downstream edge. The extent of migration over the 16 h experimental period can be seen by comparison with *a*. Scale bar, 100 μm .

autoradiography. Information about the different flow patterns was obtained in separate experiments using suspended latex spheres of 2 μm diameter.

In the cultures with unmodified lids, the medium shows the slow convective stirring that is thought to be commonly present in undisturbed cultures². Enhanced incorporation of ³H-thymidine at the wound edges is very marked in 4% or 6% serum (Fig. 2*a*). Most of the edge enhancement is contributed by cells that have migrated into the original wound area. With 8% or 10% serum, the background labelling of cells over the whole cell sheet becomes so high that the edge effect is not discernible.

In the cultures with modified lids but with the glass disk kept stationary throughout the experiment, convection currents are suppressed beneath the disk and the movement of latex spheres appears to be confined to brownian motion. At 6% serum concentration (Fig. 2*b*), the enhanced labelling of cells at the

wound edges is not so marked as in the unmodified cultures. At higher serum concentrations, background labelling beneath the disk is lower than in the unmodified cultures. The extent of cell migration into the wound area does not appear to be affected by the presence of the disk.

If the disk is rotated at 0.77 r.p.m. throughout the experiment, the latex spheres follow concentric circular streamlines, the flow beneath the disk appearing to be entirely tangential with no radial or vertical component. Extensive flow studies with enclosed rotating disks⁹ confirm that this laminar flow is to be expected with our conditions, the velocity varying linearly across the gap from zero at the Petriperm membrane to ωr at the glass disk (where ω is the angular velocity and r is the radial distance from the axis of rotation). With 6% or 8% serum concentration, we see a clear difference in the number of labelled cells at the upstream and downstream edges of the wound. At 6% serum concentration (Fig. 2*c*), the edge effect is suppressed at the upstream edge and, in most cases, it ceases to be noticeable. With 8% serum (Figs 2*d*, 3*b*), the upstream edge effect is noticeable but is still markedly smaller than the downstream edge effect. This asymmetry in labelling of the two wound edges cannot be accounted for by differences in the extent of cell migration into the wound area: the difference in labelling index (proportion of labelled cells) between sampled areas from the upstream and downstream edges within the confines of the original wound area is highly significant (8% serum, upstream: 76/685; downstream: 215/913, $\chi^2 = 41$, d.f. = 1, $P < 0.001$).

The level of serum needed to stimulate new growth beneath a stationary or rotating glass disk is higher than in the absence of the disk. It is unlikely that the disk affects gaseous exchange to the cells since the cells are grown on gas-permeable membrane. The fact that the glass disk, even when rotating, suppresses the vertical component of flow in the medium, suggests

that this reduced growth rate is compatible with the explanation of increased growth caused by shaking cultures⁴. The alternative contact hypothesis does not readily explain these effects.

In the laminar flow experiments, the difference in growth between the two wound edges shows that the normal growth response to local cell density can become distorted by flow. This difference cannot be attributed to a difference in shear stress as this is the same for both wound edges at any particular distance from the centre of rotation. In any case, the maximum shear stress in our system is only $0.012 \text{ dyne cm}^{-2}$, more than 100 times lower than the stresses reported to affect cells^{7,8}. Furthermore, the extent of cell spreading, migration and loss of contact is similar at the two edges (Fig. 3) and so it is unlikely that the difference in growth is a secondary consequence of flow affecting

these properties. The only difference in local conditions between the two wound edges is that at the upstream edge the medium flows off the cell sheet, whereas at the downstream edge it flows off the cell-free wound area. We conclude that the signal for determining cell growth is carried by the flow so that the growth response at any location is influenced by the cell density extending some way upstream. The most likely candidate for a signal of cell density that can be transferred by flow is the concentration of some soluble factor that is released or absorbed by cells. An explanation consistent with the dependence of growth on serum concentration is that the signal is a serum-derived growth-promoting factor.

We thank Patricia Dopping-Hepenstal for growing 3T3 cells and Richard Adams for computer software.

Received 18 June; accepted 6 Sept 1984.

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Immunoglobulin-like nature of the α -chain of a human T-cell antigen/MHC receptor

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Although the receptor with which T cells bind specific antigen can, like immunoglobulin, distinguish between antigens which differ only slightly in structure, it is unique in recognizing antigen only in conjunction with one of the self proteins of the major histocompatibility complex (MHC restriction)¹⁻⁴. The receptor was identified and characterized in mouse and man by using monoclonal antibodies to receptor idiotypes⁵⁻⁷, and consists of two disulphide-linked polypeptides, an acidic α -chain and a neutral to slightly basic β -chain^{8,9}. Peptide maps have shown that, like immunoglobulin, both chains vary for receptors of different specificities¹⁰⁻¹². T-cell-derived cDNA clones have recently been identified in mouse and man encoding immunoglobulin-like molecules¹³⁻¹⁵. These were identified as derived from β -chain genes through a partial N-terminal protein sequence of the β -chain isolated from a human T-cell tumour¹⁶. We have now purified the α - and β -chains of the receptor of the human T-cell leukaemia line HPB-MLT, and have determined the amino acid sequence of several tryptic peptides derived from each chain. Our results further confirm that the previously reported cDNA clones encode β -chains. The sequence of the α -chain peptides identify this as another immunoglobulin-like polypeptide chain. Particularly striking was an α -chain peptide with high homology to the conserved portion of the immunoglobulin J segment and T-cell receptor β -chains. Surprisingly, the α -chain peptides show little similarity to the sequence predicted by two overlapping putative murine α -chain cDNA clones¹⁷.

Using a monoclonal antibody to the receptor on the HPB-MLT cell line¹⁰, we purified several nanomoles of receptor trace-labelled with ¹²⁵I. The α - and β -chains of the receptor were separated and purified by two-dimensional non-reduced/reduced SDS-polyacrylamide gel electrophoresis (PAGE) as described in Fig. 1 legend. Both chains were cleaved

with trypsin and the resultant peptides were purified by reverse-phase HPLC (Fig. 2). Two β -chain peptides, designated Y and Z, were sequenced (Fig. 3). Identical sequences are predicted by the human cDNA sequence of Yanagi *et al.*¹³, further confirming this cDNA as encoding a β -chain and increasing our confidence that the other chain we had purified on the basis of its covalent linkage to this β -chain was indeed a *bona fide* α -chain.

Eight α -chain peptides were sequenced (labelled A-H in Fig. 2a); except for a few amino acids and in some cases the terminal lysine or arginine, an unambiguous sequence was obtained for each peptide. These sequences, listed in Fig. 3, represent the first reported for an α -chain. Each of the peptides was compared with all known sequences in the Dayhoff protein data base¹⁸ using the Unitary Matrix and the Mutation Data Matrix comparison programs. Obviously, because the peptides are so short each peptide showed sequence similarities to a different random set of bacterial, viral and eukaryotic proteins, but in no case was a perfect match found. However, most importantly, only homologies to immunoglobulins and other T-cell receptor chains occurred among the peptides.

The most striking similarity was found for peptide 'E', which shows clear homology to both T-cell receptor β -chain and immunoglobulin J segments (Fig. 4). The strongest homology is 75% (6/8) with the $J_{\beta 1-2}$ segment from the β -chain of the human T-cell tumour Molt-3 (refs 13, 19) and with the murine T-cell β -chain $J_{\beta 2-3}$ (ref. 20). Note that these homologies occur towards the C-terminal end of the J segments, within the strongly conserved region. This great similarity to β -chain J segments suggests that peptide E might be a particularly high yield peptide derived from some β -chain contaminating the purified α -chain. This possibility is eliminated by the absence of the peptide in the tryptic digest of HPB-MLT β -chain (Fig. 2). The identification of a J segment in the α -chain not only identifies its immunoglobulin-like nature but also predicts that the α -chain is encoded by separate V, J and C genes, and that immunoglobulin-like V-J rearrangements must occur in T cells to produce a functional α -chain gene. Confirmation of this prediction must await isolation of this gene.

Peptides F, G and H also show some similarity to immunoglobulins and other T-cell receptor sequences. Although these similarities are less striking than that of peptide E, they do strengthen the conclusion that the α -chain is an immunoglobulin-like molecule. Peptide G is worth discussing in some detail. This proline-rich peptide contains an acid-cleavable bond between aspartic acid and proline (Fig. 3). We have identified a single acid cleavage site in the HPB-MLT-derived α -chain roughly in the middle of the molecule (C.H.H., unpublished data). By analogy with immunoglobulin and other T-cell

Fig. 1 Purification of the α - and β -chains of the receptor of HPB-MLT. Lysate was prepared as described previously^{7,8,10} from 5×10^{10} HPB-MLT cells and combined with lysate prepared from 5×10^8 ¹²⁵I-surface labelled HPB-MLT cells. The lysate was incubated overnight with excess Sepharose 4B beads coupled with the anti-receptor monoclonal antibody T40/25. The bound receptor was eluted with 0.05 M diethylamine, pH 11, and immediately neutralized with Tris buffer. A portion of the eluate corresponding to 5×10^8 cell equivalents was boiled in Laemmli sample buffer²⁴ and run in a single lane of a 10% SDS-PAGE slab gel without reduction. The lane was sliced from the gel, soaked briefly in sample buffer containing 5% 2-mercaptoethanol, laid along the top of a second 10% slab gel and electrophoresed again. The gel was stained with Coomassie blue (a) and an autoradiogram of the dried gel prepared (b). The 46,000-molecular weight (46K) α -chain and 38K β -chain are indicated in each panel, with molecular weight standards positioned along the diagonal containing proteins whose molecular weight did not change on reduction. For preparation of α - and β -chain from the rest of the eluate, this analytical method was scaled up. One-fifth of the T40/25 Sepharose eluate was loaded along the entire top of a 10% slab gel and run without reduction, with cytochrome *c* added as carrier to protect the receptor from oxidation. The gel was sliced into thin horizontal strips and those containing receptor (~ 85 K), detected by radioactivity, were soaked briefly in sample buffer containing 5% 2-mercaptoethanol, loaded along the top of a second slab gel, and electrophoresed again including 0.1 mM thioglycollate in the cathodic buffer chamber²⁵. This gel was also cut into thin horizontal slices and those corresponding to the α - and β -chains were identified by radioactivity. Finally, all slices containing either α - or β -chain were combined and pulverized. The proteins were eluted with water, dialysed extensively, reduced again with dithiothreitol, alkylated with 4-vinylpyridine²⁶, concentrated by lyophilization and precipitated with acetonitrile. The overall yield from five purifications was ~ 2 -3 nmol of each chain.

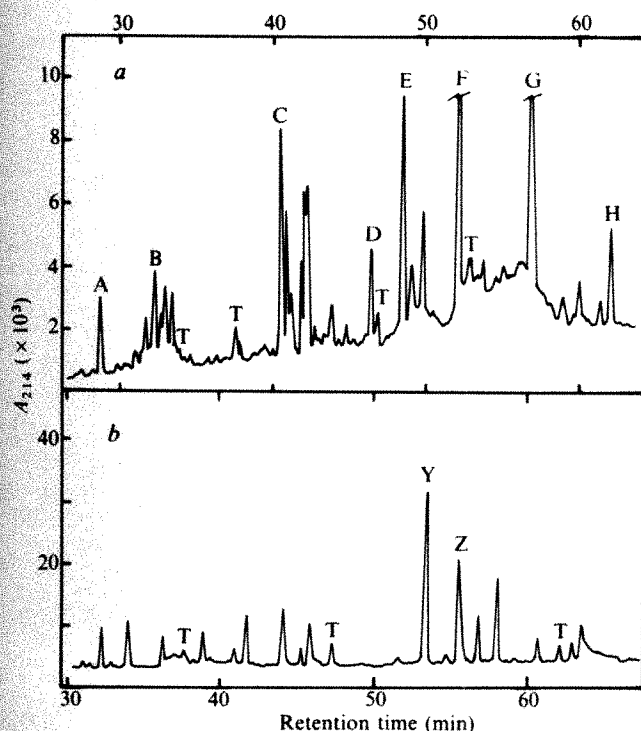
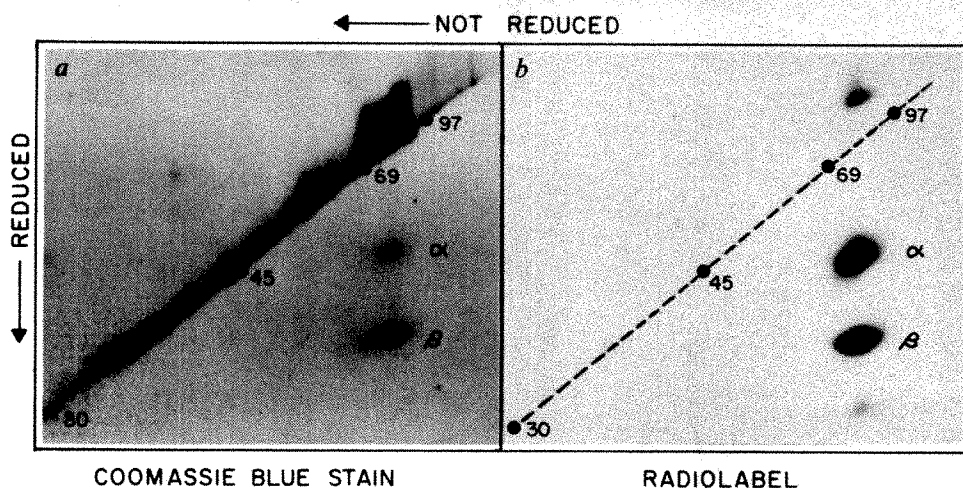


Fig. 2 Preparation of tryptic peptides from α - and β -chain. *a*, The elution pattern of the α -chain peptides. The peptides selected for sequencing are labelled A-H. *b*, The elution pattern of the β -chain peptides. The peptides selected for sequencing are labelled Y and Z. In both panels peptides known to be derived from the autodigestion of trypsin are labelled T.

Methods: Samples containing 1-2 nmol of α -chain and 2-3 nmol of β -chain were resuspended in 0.5 ml of 0.05 M ammonium bicarbonate and treated with 10% (w/w) TPCK-trypsin added as two equal aliquots over a 16-h incubation at 37 °C. The reaction was stopped by lyophilization. The lyophilized pellet was resuspended in 100 μ l of 0.1% trifluoroacetic acid (TFA) in water (solvent A). The insoluble core peptides were removed by centrifugation at 2,000g for 20 min. The soluble peptides were injected on a 4.6×250 mm Altex Ultrasphere-I.P. 5 μ C-18 reverse-phase column. The column was eluted at 1 ml min⁻¹, with an 80-min linear gradient of 0-50% solvent B (0.1% TFA in acetonitrile) in solvent A²⁷. The column effluent was monitored at 214 nm and 0.5-ml fractions collected.

receptor proteins, this would place the peptide towards the N-terminal end of the constant region. Similar proline-rich stretches occur in the beginning of the constant region of T-cell receptor β -chains^{13,15} and in many immunoglobulin light- and heavy-chain constant regions²¹. In immunoglobulins, these occur in the β -pleated sheets involved in the interaction between light and heavy chains²², and it is tempting to propose a similar structure and function in T-cell receptor α - and β -chains.

One of the most surprising results of our study is the relatively slight similarity of the α -chain peptides of the HPB-MLT receptor to the reported sequence of a proposed murine α -chain predicted from the pHDS4/203 cDNA clones isolated from a

cytotoxic T-cell clone¹⁷. Overall, the HPB-MLT peptides are no more similar to this sequence than they are to immunoglobulins or T-cell receptor β -chains. Given the extraordinary homology between mouse and human β -chains, it seems unlikely that the function of the T-cell receptor could tolerate such divergence between mouse and human α -chain sequence. Rather, one must consider the possibility that this murine cDNA sequence does not encode a receptor α -chain, but some other immunoglobulin-like molecule. Although this might seem unlikely, several properties of this protein are inconsistent with the known properties of murine α -chains. Murine α -chains are acidic proteins, with N-linked carbohydrate adding as much as

Peptide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
α chain																		
A	Leu	Val	Glu	Lys														
B	()	Asn	Ser	Ala	Val	Ala	Trp	Ser	(Ala)	Lys								
C	Asp	Ser	Asp	Val	Tyr	Ile	Thr	Asp	(Lys)									
D	Thr	Val	Leu	Asp	Met	Arg												
E	Ile	Ile	Phe	Gly	Ser	Gly	Thr	Arg										
F	Tyr	Ser	Trp	Asn	Phe	Gln	(Lys)											
G	Leu	Ser	Ile	()	Pro	Asn	Ile	Gln	Asn	Pro	Asp	Pro	Ala	Val	Tyr	Gln	Leu	(Arg)
H	Glu	Asp	Val	Glu	Gln	Ser	Leu	Phe	Leu	Ser	Val	()						
β chain																		
Y	Val	Ser	Ala	Thr	Phe	Trp	Gln	Asn	Pro	()								
Z	Ala	Lys	Pro	Val	Thr	Gln	Ile	Val	Ser	Ala	Glu	Ala	Trp	Gly	()			

Fig. 3 Amino acid sequence of the α - and β -tryptic peptides. Fractions from the HPLC, containing individual peptides, were pooled and evaporated to dryness by lyophilization. Sequences were determined on an Applied Biosystems gas-phase sequencer using either the '01' or '02' chemistry with TFA conversion of the thiazolinones to phenylthiohydantoin (PTH). Analysis of the PTH-derivatives produced was carried out on a Waters Novapak C-18 reverse-phase column eluted with a complex gradient of 16% acetonitrile in 40 mM sodium acetate, pH 5.0 (solvent A) and 60% isopropyl alcohol in water (solvent B). PTH-derivatives were monitored at 254 nm. Residues in parentheses indicate uncertain assignments. Open parentheses indicate that no assignment could be made at that position, although when at the end of a peptide the open parentheses indicate either a lysine or arginine.

Human	Peptide E	I	I	F	G	S	G	T	R
	J β 1-2	Y	T	F	G	S	G	T	R
	J β 1-1	A	F	F	G	Q	G	T	R
	J β 1-1	V	F	F	G	K	G	T	R
	J β 1-3	L	Y	F	G	E	G	S	R
	J β 1-4	L	F	F	G	H	G	T	K
	J β 1-5	P	L	F	G	E	G	T	R
	J β 1-6	L	Y	F	A	A	G	T	R
Murine	J β 2-1	Q	F	F	G	P	G	T	R
	J β 2-2	L	Y	F	G	E	G	S	K
	J β 2-3	L	Y	F	G	S	G	T	R
	J β 2-4	L	Y	F	G	A	G	T	R
	J β 2-5	Q	Y	F	G	P	G	T	R
	J β 2-7	Q	Y	F	G	P	G	T	R
	J pHDS4/203	K	V	F	A	E	G	T	K
	J β consensus	-	-	W	G	Q	G	T	L
Human	J κ consensus	-	-	F	G	Q	G	T	K
	J λ consensus	-	-	F	G	G	G	T	K

Fig. 4 Comparison of the sequence of peptide E of the α -chain with that of J segments of other T-cell receptor chains and of immunoglobulins. The sequence of peptide E of the receptor α -chain of HPB-MLT is shown aligned with portions of the J segment of human and mouse β -chains^{13,19,20,28}, the J segment predicted by the pHDS4/203 cDNA clones¹⁷, and consensus J segments for human immunoglobulins²¹. Identical residues are boxed. The standard one-letter amino acid code is used: A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.

10,000–15,000 to their molecular weight^{5,8,9,23}. The pHDS4/203 cDNA encodes a basic protein with no sites for N-glycosylation. Furthermore, peptide maps of receptor α -chains have indicated variation between T-cell clones of different specificities^{10–12}. However, the expression of the genes detected by pHDS4/203 seems to involve only one V region and one J segment. Further

experiments will be necessary to resolve these apparent discrepancies.

We thank Craig R. Miles for technical assistance in obtaining the N-terminal amino acid sequences, Jim Leibson, Ella Kushnir and Janice White for other valuable technical help, Dr Ralph Kubo for help in the preparation of the immunosorbent beads and for sharing his protein chemistry experience with us, Dr George Tarr of the University of Michigan for his advice in our early attempts to sequence these proteins, and Kelly Bakke, Ruth Roberts and Edna Squillante for secretarial help. This work was supported by USPHS grants CA-36700 (to J.H.F.) and AI-17134 (to J.W.K. and P.M.) and American Cancer Society grants IM-49 (to J.W.K. and P.M.) and FRA-218 (to J.W.K.).

Received 3 September; accepted 27 September 1984.

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Cell-surface antigens expressed on L-cells transfected with whole DNA from non-expressing and expressing cells

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We have shown previously¹ that transfection of mouse L-cells with DNA from JM, a human T-cell line expressing certain T-cell differentiation antigens, yields stable transfectants expressing one or another of these antigens. The identities of the antigens were confirmed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. We now report that our procedure—co-transfection² with the chicken thymidine kinase gene (*tk*) and whole cellular DNA, selection with hypoxanthine-aminopterin-thymidine (HAT)³, and staining of the cells with fluorochrome-conjugated monoclonal antibodies and fluorescence-activated cell-sorter (FACS) selection—yields transfectants expressing a variety of cell-surface molecules (19 of 21 investigated), most at a frequency of about one per 10³ Tk⁺ transformants. Of these, 9 of 12 were transferred and expressed as readily using DNA from cells which did not express the cell-surface antigens as from tissues or cells that did express them.

We performed Tk co-transfections with DNA from human placenta, kidney, a renal cell carcinoma derived from the same individual as the kidney, and LCL-721, a human B-lymphoblastoid cell line. None of these tissues expresses serologically detectable Leu-1 or Leu-2, except LCL-721, which expresses Leu-1; in none is Leu-2 messenger RNA detectable by Northern blotting using cloned Leu-2 complementary DNA as probe²⁸. DNA from JM, which expresses these genes, served as a positive control.

Tk⁺ transformants were selected by FACS for expression of Leu-1 or Leu-2. As positive controls, we selected transfectants expressing HLA class I antigens or human transferrin receptor, as these are constitutively expressed in all of the tissues we studied. We recovered Leu-2⁺ transfectants from all of the

Table 2 Murine lymphoid antigens detected on transfected L-cells using monoclonal antibodies

Antigen	Tissue source of DNA			
	Spleen	Kidney	Muscle	L(Tk ⁻)
Ly-1	3/3*	2/2	2/2	5/6
Ly-2	2/3	2/2	2/2	4/6
BLA-2	2/3	2/2	2/2	3/6
ThB	3/3	2/2	2/2	ND
FcR(ref. 26)	2/3	0/2	1/2	ND
Thy-1	1/3	2/2	1/2	ND
L3T4a(ref. 27)	1/3	ND	1/2	4/6
H-2 ^d	3/3	2/2	ND	ND

* No. of dishes yielding positive transfectants/total no. of dishes analysed.

human DNA sources, at a frequency of about one per 10³ Tk⁺ transformants (Table 1). We also recovered, though less frequently, transfectants expressing either Leu-1 (from both antigen-expressing and non-expressing sources) or human transferrin receptor.

In our original work with JM DNA, one of the Leu-2⁺ transfectants expressed very high, but unstable, levels of surface Leu-2. The gene for Leu-2 was highly amplified in this transformant^{4,28}. We have now found that 11 of 20 independent Leu-2⁺ transfectants exhibit similarly high and heterogeneous levels of Leu-2. In 10 of these cases, further rounds of selection for the most brightly staining cells have yielded at least a 4-fold increase in antigen expression, resulting in cells that are now over 20-fold brighter than 'normal', stable Leu-2⁺ transfectants. In some cases, we have confirmed Leu-2 gene amplification by quantitative filter hybridization of RNA⁵ and DNA⁶ to a cloned Leu-2 gene probe²⁸.

To further our study using multiple organ and tissue sources and to determine how many of the antigens for which we had available antibodies could be transferred and expressed, we extended our work to mouse DNA transfections. We studied 11 murine cell-surface antigens. Nine are believed to be monomeric glycoproteins. One, Lyt-2, occurs primarily as a heterodimer⁷. The two chains may be encoded by one gene or

Table 1 Human antigens detected on transfected mouse L-cells using monoclonal antibodies

Antigen	DNA donor				
	Placenta	Kidney	Renal cell carcinoma	LCL-721 (B-lymphoblastoid)	JM (T-cell lymphoma)
HLA (W6/32)	ND	3/6†	6/6	4/4	2/2
Leu 2	6/6	3/6	1/6	7/8	3/6
(Leu 2)*	(4/6)	(1/3)	(1/1)	(3/7)	(2/3)

Isolation of transfectants expressing novel cell-surface antigens: L(Tk⁻) cells²² were grown in 100-mm tissue culture dishes (Lux) in Dulbecco's modified Eagle's medium with 10% fetal calf serum. DNA was prepared from various tissues as described previously²³. Briefly, the tissues were pulverized under liquid nitrogen, dissolved in 1% sarkosyl, 50 mM EDTA, 0.2 mg ml⁻¹ proteinase K (Merck), followed by treatment with RNase, extraction with phenol followed by chloroform, and caesium chloride equilibrium gradient centrifugation. The purified DNA was recovered (and sterilized) by ethanol precipitation and dissolved in 10 mM Tris-HCl, 1 mM EDTA. Transfections were carried out using a modification of the protocol of Wigler *et al.*²⁴. In most cases the DNA was sheared before precipitation by one passage through a 23-gauge needle. In all cases, 1 µg of Tk-bearing plasmid (the chicken thymidine kinase gene, cloned into the plasmid pBR322 and propagated in the host *Escherichia coli* GM48 (*dam* *mec*)²⁵ was used with 20 µg of genomic ('carrier') DNA per dish of 10⁶ cells. Transfectants were selected for expression of Tk genes by growth in HAT³. They were then selected for expression of specific antigens by staining with fluoresceinated monoclonal antibodies and FACS sorting, as described elsewhere¹. Initial dishes of transfectants were pooled, if necessary, to form dishes of 1,000–1,500 Tk⁺ clones each. These were then collected, and each dish was divided into aliquots of 10⁶ cells, one aliquot for each reagent to be screened. Staining was done at nominally saturating concentrations of antibody, in sterile 96-well V-bottom culture trays. In the initial round of sorting, cells falling within arbitrary 'gates' representing the positively staining cells were sorted into pots and then regrown for further analysis. In some cases it proved possible to clone positively staining cells directly from the initial culture. In most cases it was necessary to enrich with one or more rounds of 'pot-sorting' for bright cells before it became feasible to clone true positives into individual wells.

W6/32 is an anti-HLA framework monoclonal antibody that is specific for HLA heavy chains even when they are in association with mouse β₂-microglobulin^{20,21}. ND, not determined.

* Amplified phenotype (no. amplified/no. positive).

† No. of dishes yielding positive transfectants/total no. of dishes analysed.

Table 3 Expression of mouse genes in transfected L-cells requires mouse DNA

DNA source	Human Leu-2a	Human NGF receptor	Antigen Rat NGF receptor	Mouse 30-E2	Mouse L3T4a
PC12(rat)	ND	ND	5/8*	0/8	0/8
A875(human)	4/6	1/6	ND	0/6	0/6
Peripheral blood lymphocytes (human)	6/6	ND	ND	0/6	0/6

PC12 is a rat chromocytoma expressing NGF receptor; A875 is a human melanoma expressing NGF receptor but not Leu-2. Transfectant cultures were subjected to at least four rounds of sorting and regrowth before being typed as negative. In previous experiments with 30-E2 and L3T4a, no more than two rounds of FACS selection had ever been required to obtain cultures that were >90% enriched for positive cells, when there were positives present in the starting dishes of transfectants. We note that the results with A875 provide another example of efficient transfer of Leu-2 using DNA from a non-expressing source.

* No. of dishes yielding positive transfectants/total no. of dishes analysed.

by closely linked genes. In the case of H-2, the host cell⁸ or the culture medium serum⁹ can supply β_2 -microglobulin to complement the heavy chains encoded by the donor DNA, and permit surface expression.

We used DNA from BALB/c mice, as they differ at several serologically detectable loci from the mouse strain (C3H) from which L-cells were derived¹⁰. Because all of the antigens tested are present on at least 10% of spleen cells^{11,12}, we used spleen DNA as our positive standard. None of these antigens is known to be expressed on any of the other tissues used. The various DNA sources differed little in yield of transfectants for 8 of the 11 antigens tested (Table 2). For the other three antigens (T200, BLA-1 and 30-Fl, not tabulated), we recovered only one transfectant, preventing meaningful comparisons.

For heuristic purposes, we also used DNA from the L(Tk⁻) line itself. We used only those antibodies with which we had previously most readily detected transfectants. We recovered L-cell transfectants positive for the target antigens as frequently with L-cell DNA as with the other DNAs (Table 2).

It is conceivable that the murine antigens are produced by L-cell genes that are active in rare variants in normal L-cell cultures, or which are activated by the transfection process. We therefore transfected L-cells with human or rat DNA and looked for cells expressing murine antigens. We did not isolate any transfectants expressing the murine antigens tested, although we did obtain transfectants expressing Leu-2 or rat nerve growth factor (NGF) receptor at expected frequencies (Table 3). Furthermore, in the case of Ly-1, Lyt-2 and H-2 antigens, the BALB/c DNA-derived transfectants stain with antibodies specific for the donor allotype, showing directly that they express transferred, and not endogenous, genes.

Two antigens for which we obtained transfectants were Lyt-2 (see Table 2) and 30-Fl (data not shown). In the normal animal, each is associated with another cell-surface determinant (Lyt-2 with Lyt-3 (ref. 11) and 30-Fl with BLA-1 (R. Hardy, unpublished)). None of the Lyt-2⁺ transfectants stain with anti-Lyt-3, nor do they contain the Lyt-3 polypeptide when examined by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. However, the one 30-Fl⁺ transfectant isolated also expresses BLA-1. As co-transfer of two unlinked genes is very unlikely¹³, and in light of their association on normal cells, we propose that 30-Fl and BLA-1 are encoded by one gene or by two very tightly linked genes.

The BLA-2⁺ transfectants expressed extremely high levels of antigen, enabling us to perform an initial characterization of the molecule. We immunoprecipitated a 94,000 molecular weight protein consisting of one polypeptide chain. This molecule has now been identified on various murine B-cell lines known from staining studies to be BLA-2⁺ (R. Hardy and G. Calicchio, unpublished).

We believe this to be the first demonstration that several genes can be stably transferred in active form using DNA from tissue sources not expressing those genes. Further, in the case of most of the genes we analysed, DNA from non-expressing sources

appears to be as efficient in transfection as DNA from expressing tissues.

Other studies¹⁴⁻¹⁶, using cloned genes, show an inverse correlation between the activity of genes in gene transfer and their degree of cytosine base methylation. The means by which the genes in our study were originally turned off may not depend on this type of modification, or they may be insufficiently modified to keep them turned off in L-cells. We have readily recovered Leu-2⁺ transfectants using the highly methylated¹⁷ DNA from human sperm. However, we have not recovered any Leu-2⁺ transfectants using DNA from certain human choriocarcinoma cell lines (S. Alberti, C.H. and L.A.H., in preparation); these are also the only DNA sources we have used which do not express antigens of the major histocompatibility complex. We are now investigating whether there is a special mechanism for turning off genes in these cells.

Enhancer sequences have been shown to be important in the expression of certain cloned genes in transfection systems^{18,19}. These *cis*-acting elements increase transcription rate and accuracy, and seem to act in a highly tissue-specific manner. The stringent tissue-specificity attributed to the expression of certain differentially expressed genes (for example, immunoglobulins) in gene-transfer systems does not seem to hold in L-cells for the genes we have transferred using total cellular DNA.

We thank Dr R. R. Hardy for purifying and fluorescein-conjugating most of the anti-mouse antibodies used in this work, and Drs F. Fitch, J. Unkeless, L. Lanier, P. Jones and E. Shooter for gifts of reagents. This work was supported in part by NIH grants CA-04681, GM-28428 and GM-17367.

Received 8 May; accepted 21 August 1984.

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Evidence for two distinct *c-src* loci on human chromosomes 1 and 20

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A number of proto-oncogenes have recently been localized to the chromosomal segments that are the breakpoints in the specific rearrangements noted in human malignant diseases¹. Moreover, rearranged forms of several proto-oncogenes have been identified in malignant cells; in several instances, the proto-oncogene has undergone an alteration as a result of a nonrandom chromosomal rearrangement²⁻⁴. One proto-oncogene that has yet to be associated with human neoplastic disease is *c-src*, the cellular homologue of the transforming sequence of Rous sarcoma virus (RSV). By somatic cell hybridization, *c-src* has been mapped to chromosome 20, but its precise location was not determined⁵. We have now mapped this gene by using *in situ* hybridization of the cloned human *c-src* probe to human mitotic chromosomes. We report here that the human genome contains two loci with strong homology to the coding regions of this oncogene, at 1p34-p36 and 20q12-q13. It is noteworthy that these chromosomal regions are frequently involved in the structural rearrangements observed in haematological malignant diseases⁶⁻⁸.

In situ hybridization⁹ of a ³H-labelled *c-src* probe containing genomic *c-src* sequences spanning exons 10-12 (plasmid pHu-1-c) resulted in specific labelling of two chromosomal regions, the distal short arm of chromosome 1 and the long arm of chromosome 20. Figure 1 shows the distribution of labelled sites in 100

metaphase cells examined. Of these cells, 40 (40%) were labelled on region 1p3, bands p34-p36, of one or both chromosomes 1; these sites represented 13.5% (68 of 505) of all labelled sites ($P < 0.005$). On chromosome 20, 34 metaphase cells (34%) exhibited label on the mid- to terminal segment of 20q. Fifty labelled sites, or 9.9% of all labelled sites, were noted on 20q12-q13 ($P < 0.005$). The distribution of grains from labelled chromosome 1 ($n = 65$) and labelled chromosome 20 ($n = 51$) indicates significant clustering of grains to bands 1p34-p36 and to bands 20q12-q13 (Fig. 2). A small cluster of grains was observed at band 9q34 ($P < 0.005$, 1.8% of labelled sites), suggesting that cross-hybridization to *c-abl* had occurred¹⁰. When the stringency of hybridization was increased (hybridized slides washed extensively at 42 °C rather than 39 °C), labelling was not observed in this region. However, specific labelling of 1p34-p36 (9.6% of labelled sites) and 20q12-q13 (7.9% of labelled sites) was maintained under more stringent conditions (60 metaphase cells examined). *In situ* hybridization with an 800-base pair *Pvu*II fragment containing *v-src*-specific sequences derived from the Schmidt-Ruppin strain of RSV (plasmid pPvuIIIE)¹¹ confirmed the presence of these two loci (data not shown). Although the signal was much weaker than that observed with the human *c-src* probe, a clustering of grains was again observed on 1p34-p36 and 20q12-q13.

RSV is one of the most highly oncogenic of the known retroviruses. Infection of avian fibroblasts with RSV results in a high degree of malignant transformation; this transforming capability of RSV is mediated by the *v-src* sequences¹². Interestingly, infection of cultured mouse bone marrow with RSV results in increases in stem cell proliferation and in self-renewal, two properties associated with myeloproliferative disorders¹³.

We have now regionally mapped the proto-oncogene *c-src* to two sites in the normal human karyotype by using *in situ* hybridization. The fact that the two loci hybridized to both the

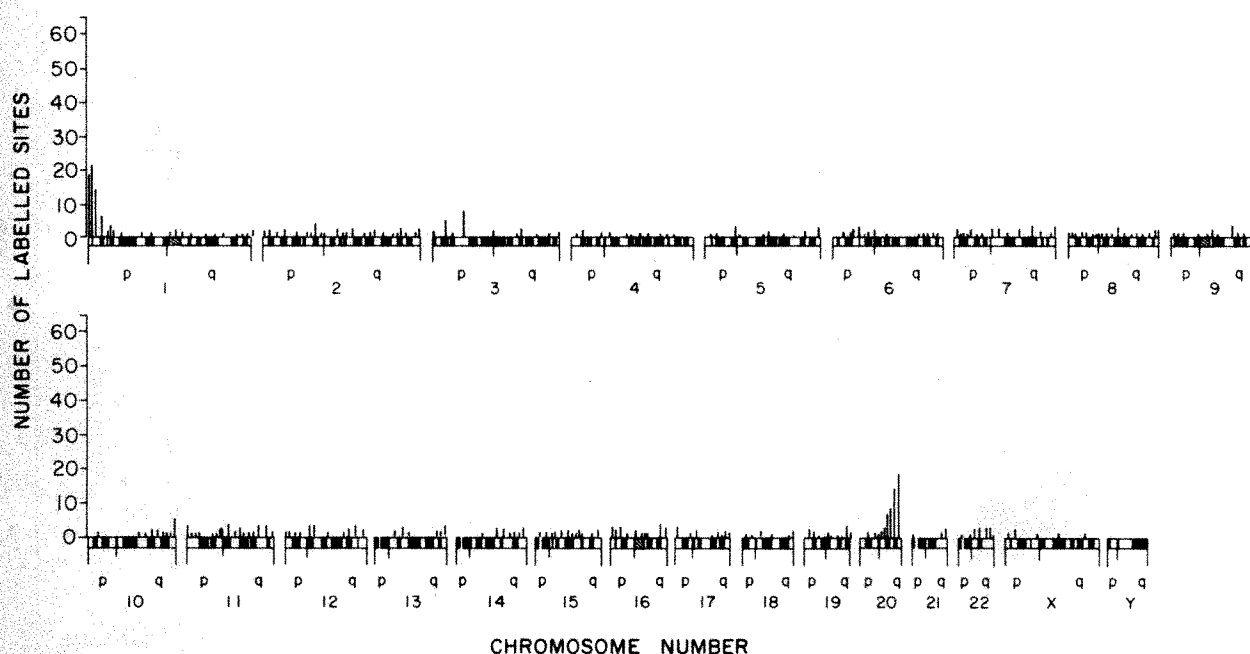


Fig. 1 Sub-localization of the human *c-src* gene to two human chromosomes. Distribution of labelled sites in 100 metaphase cells identified significant clustering of grains on regions p3 and q1 of chromosomes 1 and 20, respectively. Human metaphase cells prepared from phytohaemagglutinin-stimulated peripheral blood lymphocytes were hybridized with the ³H-labelled pHu-1-c plasmid DNA which contained a 1.7-kilobase *c-src*-specific insert, encompassing exons 10, 11 and 12 of the human genomic *src* sequence, cloned in pUC8. Radiolabelled probes were prepared by nick translation of the entire plasmid using all four ³H-labelled dNTP to specific activity 5×10^7 – 1×10^8 d.p.m. per μ g. Metaphase cells were hybridized at 10 or 30 ng probe per μ l for 16 h at 37 °C, exposed to autoradiographic emulsion for 8–15 days, and G-banded with the Wright's staining method as described by Harper⁹. The following modifications of the method of Harper were used. Chromosomal DNA was denatured by immersion of slides in 70% formamide in 0.6 $\times$ SSC (0.09 M NaCl/0.009 M trisodium citrate) at 70 °C, pH 7.0. Radiolabelled probe was hybridized in 50% formamide/2 $\times$ SSCP (0.3 M NaCl/0.03 M trisodium citrate/0.04 M NaPO₄, pH 5.0)/10% dextran sulphate/3 mM Tris–0.3 mM EDTA (pH 7.0). After hybridization, the slides were washed extensively in 50% formamide/1 $\times$ SSC, 39 °C, pH 7.0, followed by washes in 2 $\times$ SSC and by ethanol dehydration. For improved chromosome banding, autoradiographs were destained with 95% ethanol-HCl (0.25%, 10 M HCl) and restained, either once or twice.

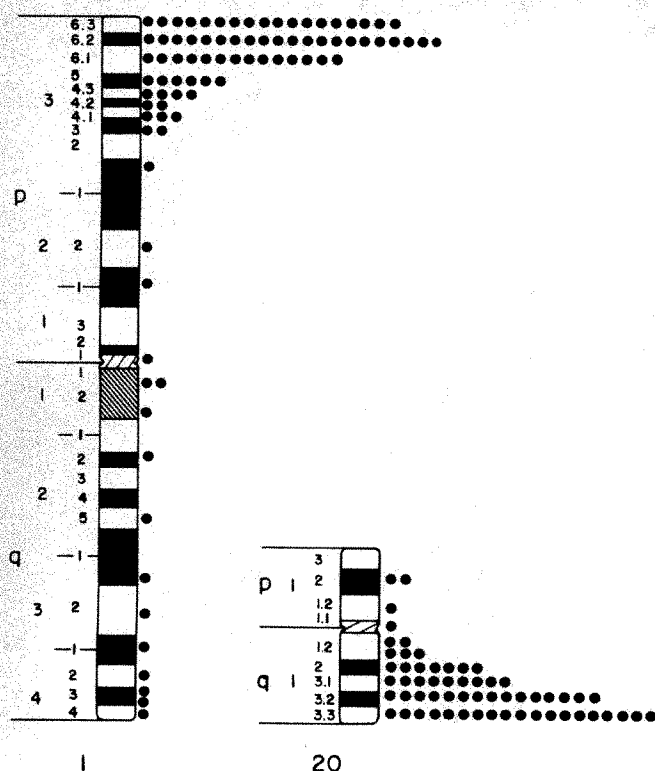


Fig. 2 Distribution of labelled sites on 65 and 51 labelled chromosomes 1 and 20, respectively. Of 86 total sites on chromosome 1, 68 (79%) were located on 1p34-pter. Of 59 labelled sites on chromosome 20, 50 (84.7%) were noted on q12-qter.

v-src and *c-src* probes suggests that the homology lies within the coding regions of the genes rather than in the intervening sequences that are absent in the *v-src* probe. With man-mouse somatic cell hybrids, sequences homologous to *v-src* had previously been mapped to chromosome 20⁵ and, recently, to its long arm (A. Y. Sakaguchi, personal communication). Southern blot analysis of less strongly hybridizing bands that did not segregate with chromosome 20⁵, suggested that additional loci may exist. Moreover, two related DNA clones corresponding to two separate *src* genes have been isolated from a human genomic library (R. Parker, G. Mardin, R. Lebo, H. Varmus and J. M. Bishop, in preparation), and were assigned to chromosomes 1 and 20 by hybridization of specific probes to dot blots of human chromosomes isolated by fluorescence activated sorting. Whether both of these genes are transcribed is not known; however, human cells contain two related, but distinct, protein forms of pp60^{c-src} (ref. 14).

Translocations involving bands p34-p36 of chromosome 1 have been noted frequently in haematological neoplasia and in solid tumours^{7,8}. We have observed rearrangements involving these bands in 5% of patients with acute non-lymphocytic leukaemia or a myelodysplastic syndrome, and in 10% of patients with non-Hodgkin's lymphoma (E. Brown, J.D.R. and M. M. Le B., unpublished results). Nonrandom deletions of 1p with a breakpoint between 1p13 and 1p36 have been identified in neuroblastomas¹⁵ and in malignant melanomas¹⁶. Abnormalities of chromosome 20 have been observed in a number of patients with haematological malignant diseases. Although loss of one chromosome 20 and translocations involving 20q have been reported, a more common abnormality is a deletion of the long arm of the chromosome. Such a deletion has been noted in patients with acute non-lymphocytic leukaemia or myelodysplastic syndrome, as well as in those with polycythaemia vera^{6-8,17,18}. This abnormality has been described as a terminal deletion involving band q11 (ref. 18). However, at the light

microscopy level, we cannot exclude the possibility that the deletion is actually an interstitial one with breakpoints at q11 and q13. *In situ* hybridization of *c-src* to malignant cells with a del(20q) would provide a definitive answer. We are now investigating these possibilities, along with an analysis of cells with rearrangements of chromosome 1. In view of our findings that *src*-specific sequences are localized to two human chromosomes that are frequently involved in rearrangements, a role for *c-src* in the pathogenesis of human neoplastic diseases should be considered. In this regard *c-src*-related sequences have been found to be expressed in malignant cells of two patients with haematopoietic neoplasms¹⁹.

We thank J. Michael Bishop for the gift of the cloned *src* probes and for valuable discussion, Diane Roulston for technical assistance, and Nanci Carrothers for help in the preparation of the manuscript. This work was supported in part by US Department of Energy, contract DE-AC02-80EV 10360, PHS grant CA 16910 from the National Cancer Institute, by the University of Chicago Cancer Research Foundation (J.D.R., M.D.), ACS grant IN-41-W-14 (M.D.), and by a Leukemia Research Foundation Grant (M.D.). M.M.L. is a Fellow of the Leukemia Society of America. C.A.W. is a Fellow of the Damon Runyon-Walter Winchell Cancer Foundation.

Received 6 June; accepted 16 August 1984.

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Biological properties of human *c-Ha-ras1* genes mutated at codon 12

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Vertebrate genomes contain proto-oncogenes whose enhanced expression or alteration by mutation seems to be involved in the development of naturally occurring tumours^{1,2}. These activated genes, usually assayed by their ability to induce the malignant transformation of NIH 3T3 cells, are frequently related to the *ras* oncogene³⁻¹⁰ of Harvey (*Ha-ras*)¹¹ or Kirsten (*Ki-ras*)¹² murine sarcoma viruses, or a third member of this family (*N-ras*)¹³⁻¹⁵. Activation involves point mutation which often affect codon 12 (refs 16-26) of the encoded 21,000-molecular weight polypeptide (p21). To provide insight into structural requirements involved in p21 activation, we have now constructed 20 mutant *c-Ha-ras1* genes by *in vitro* mutagenesis, each encoding a different amino acid at codon 12. Analysis of rat fibroblasts transfected with these altered genes demonstrates that all amino acids except glycine

(which is encoded by normal cellular *ras* genes) and proline at position 12 activate p21, suggesting a requirement for an α -helical structure in this region of the polypeptide. The morphological phenotype of cells transformed by the activated genes can, however, depend on the particular amino acid at this position.

The human c-Ha-*ras*1 gene is contained on a genomic 4.7-kilobase (kb) *Bam*HI-*Sph*I DNA fragment¹⁷ and its coding region is distributed across four exons¹⁹. To generate codon 12 mutants, a DNA fragment encompassing exon 1 was cloned into bacteriophage M13 vector mp10 (ref. 27) and exhaustive mutagenesis at codon 12 was performed using techniques similar to those described by Zoller and Smith²⁸. The oligodeoxynucleotides for introducing the desired mutations were synthesized²⁹ in three pools, each pool containing oligomers designed to alter the sequence of codon 12 by one, two or three nucleotides, respectively (Fig. 1). The few mutants not identified in this manner were obtained by repeating the mutagenesis and screening with individually synthesized oligomers.

All mutations so generated were inserted by *in vitro* recombination back into the c-Ha-*ras*1 gene (Fig. 2). This strategy was used to minimize the possibility of obtaining transformed cell clones due to the significant overexpression of otherwise non-transforming genes which can occur when strong, heterologous promoters are used³⁰. To assess the transformation potential of the 20 c-Ha-*ras*1 plasmids, each was individually transfected onto contact-inhibited Rat-1 cells³¹ by calcium phosphate-mediated DNA transfection in the presence of the Tn5-encoded phosphotransferase (*neo*) gene^{32,33}; cells incorporating the exogenous DNA were selected in the presence of the antibiotic G418 (refs 32, 33). Cotransfected markers usually become incorporated together into recipient cells^{34,35} such that individual G418-resistant colonies could be examined for the expression of the Ha-*ras* genes independently of the morphological phenotype of the cells. In this manner we could directly confirm that cells co-transfected with non-transforming c-Ha-*ras*1 genes indeed expressed the encoded polypeptide.

When co-transfected with most of the mutant *ras* genes, 80–90% of the G418-resistant colonies contained cells which

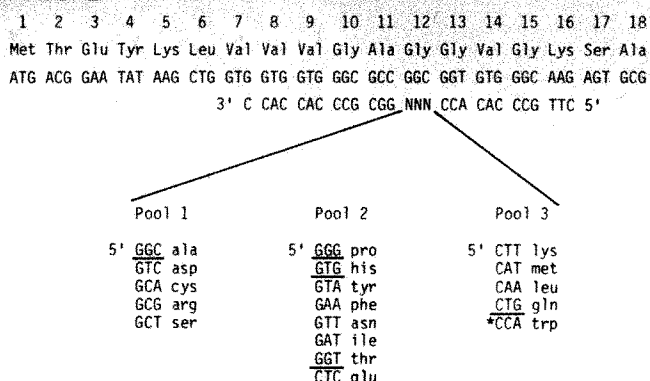


Fig. 1 Mutagenesis of c-Ha-*ras*1 codon 12. The first 18 codons of the proto-oncogene are shown, as carried on the recombinant M13 phage mp10/*ras*-Gly 12. For mutagenesis, three oligomer pools of the sequence 5'-CTTGCCACACCCNNNGGCGCCCA-CCACC-3' were synthesized by a phosphotriester approach²⁹. The actual nucleotides occupying the NNN positions of the three pools are shown; these were designed to alter the codon 12 sequence by one, two or three nucleotides, respectively. To reduce the complexity of pool 2, the oligomer changing glycine to tryptophan was included in pool 3 (see asterisk).

Methods: Oligomers (in pools or individually) were phosphorylated using rATP and polynucleotide kinase, and annealed to single-stranded mp10/*ras*-Gly DNA. Annealings (10 μ l; 10 mM Tris-HCl pH 7.5, 7 mM MgCl₂, 0.1 mM EDTA, 50 mM NaCl) containing 0.1 pmol of template DNA and 0.1 pmol of phosphorylated oligomer were incubated for 5 min at 55 °C. *In vitro* DNA synthesis was initiated by adding 40 μ l of 50 mM Tris-HCl pH 7.5, 7 mM MgCl₂, 0.1 mM EDTA, 1 mM ATP, 100 μ M each of dATP, dGTP, dTTP and dCTP, 5 U *E. coli* DNA polymerase I, large fragment (Boehringer Mannheim) and 400 U T4 DNA ligase (New England Biolabs). After incubation for 3 h at 37 °C, reaction mixtures were extracted with phenol, DNA was precipitated with EtOH, and 2–4% of the DNA was used to transform *E. coli* strain JM103 made competent for DNA uptake. Transformation mixtures (300 μ l) were spread onto bacterial plates (150 mm), yielding several hundred phage plaques. To screen for mutants, these plates were probed by *in situ* hybridization on duplicate nitrocellulose filter-lifts⁴⁵. One set of filters was probed with the oligonucleotide 5'-CTTGCCACACCCGCGGCCAC-3', fully complementary to the original sequence (coding for glycine) and other with a mutant-specific oligonucleotide or oligomer pool. Oligonucleotides were phosphorylated to a specific activity of $\sim 10^7$ d.p.m. per pmol with [γ -³²P]ATP (ICN, crude >4,000 Ci mmol⁻¹) and polynucleotide kinase (NEN). Hybridization was overnight using 10⁷ c.p.m. per filter. To screen for the original sequence, annealings were in 5 $\times$ SSC, 45% formamide, 50 °C and excess radioactivity was washed off in 2 $\times$ SSC, 0.1% SDS at room temperature, followed by 15 min at 50 °C. Conditions for detecting mutants were as follows. For mutants derived from pool 1, a set of 13-mers was used as a probe (5'-NNNGGCGCCCA-3', with NNN represented by GGC, GTC, GCG and GCT). This oligomer mixture was hybridized at 20 °C in 5 $\times$ SSC, 30% formamide, and filters were washed in 2 $\times$ SSC, 0.1% SDS at the same temperature. To probe for mutants derived from pools 2 and 3, oligomer mixtures from these pools were hybridized in 5 $\times$ SSC, 40% formamide at 60 °C and filters were washed in 2 $\times$ SSC, 0.1% SDS at 20 °C followed by a 30-min incubation at 30 °C. All filters were blotted dry after the last wash and exposed to X-ray film for several hours at -70 °C using an intensifying screen. For those mutants not obtained by using pools 1, 2 and 3 (see underlined codons), the following oligomers were synthesized individually and served as mutagenesis primers as well as mutant-specific probes:

5'-CTTGCCACACACGCGGCCCA-3' (Ala)
GGG (Pro)
GTG (His)
GGT (Thr)
CTG (Gln)

To probe for mutants, hybridization was in 5 $\times$ SSC at 20 °C and filters were washed in 5 $\times$ SSC at 20 °C, incubated in 3 M trimethylammonium chloride at 55 °C (W. Wood, unpublished) and briefly rinsed in 2 $\times$ SSC at 20 °C. Phage identified by hybridization as carrying an altered 12th codon were grown in small cultures (2 ml), and DNA was isolated and characterized by dideoxy sequencing^{46–48}.

Table 1 Transformation properties of *ras* mutants

Position 12 codon	Activated genes	Focus formation	Agar growth
Gly	c-Ha- <i>ras</i> 1 (refs 16–19), c-Ki- <i>ras</i> 2 (ref. 41), N- <i>ras</i> (ref. 15)	—	—
Ile		+++	+
Val	T24/EJ Ha- <i>ras</i> 1 (refs 16–19), SW480 Ki- <i>ras</i> 2 (ref. 21)	+++	+
Leu		+++	+
Ser	v-Ki- <i>ras</i> (ref. 12)	++	+
Thr		+++	+
Ala		++	+
Met		++	+
Cys	CaLu Ki- <i>ras</i> 2 (refs 20, 21) PR371 Ki- <i>ras</i> 2 (ref. 23)	++	+
Tyr		++	+
Phe		++	+
Trp		++	+
Pro		—	—
Lys	v-BALB- <i>ras</i> (ref. 17)	+	+
Arg	v-Ha- <i>ras</i> (ref. 11), v-Ra- <i>ras</i> (ref. 26), A1698, A2182 Ki- <i>ras</i> 2 (ref. 24)	+++	+
His		++	+
Asp	134-51 Ha- <i>ras</i> 1 (ref. 22)	++	+
Glu	NMU-Ha- <i>ras</i> (ref. 25)	++	+
Asn		++	+
Gln		+	+

Focus formation was determined by the ability of *ras*-cotransfected Rat-1 cells to form foci (see Fig. 3 legend). Agar growth was assessed by plating individual *ras*-cotransfected subclones, or cotransfected mass cultures, into soft agar⁵¹ at two densities (4×10^3 and 2×10^4 per 60 mm dish) and scoring colonies of >30 cells after 2–3 weeks.

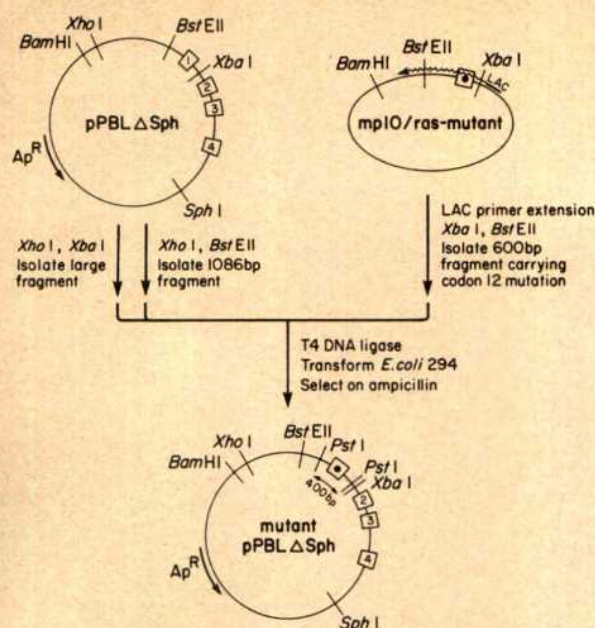


Fig. 2 Construction of *c-Ha-ras1* genes substituted at codon 12. Plasmid *pPBLΔSph*, a derivative of *pPBL116-4* (ref. 19) lacking the 1.7-kb *SphI*-*BamHI* region (containing repeated elements)¹⁹ of the 6.5-kb *BamHI* fragment, was cleaved with endonucleases *XhoI* and *XbaI*. The larger fragment (6.7 kb) containing sequences of the bacterial plasmid pBR322 and *ras* exons 2, 3 and 4, together with the presumed transcriptional terminator of the *c-Ha-ras1* gene, was isolated from a 6% polyacrylamide gel. The 1,086-bp *XhoI*-*BstEII* fragment containing the prelude region of *c-Ha-ras1* (ref. 19) was similarly isolated. DNA carrying the first exon of *c-Ha-ras1* mutated in codon 12 was obtained by gel isolation of a 600-bp *BstEII*-*XbaI* fragment cleaved from *in vitro* synthesized DNA after priming with the 17-mer LAC primer⁴⁶ on the *mp10/ras* mutant phage DNAs. The *BstEII*-*XbaI* fragment with the valine codon was isolated from plasmid pT24-10 (ref. 19). A three-component ligation with these DNA fragments yielded the mutant variants of plasmid *pPBLΔSph* used for transfections. To ensure that each mutant plasmid carried the correction codon substitution, plasmids were screened for the absence of a *NaeI* restriction site spanning codon 12 (refs 16, 17), and a 400-bp *PstI* fragment containing exon 1 was isolated from each plasmid DNA and analysed by DNA sequencing.

were not contact-inhibited, unlike control cells which received only the *neo*-resistance plasmid (see Table 1). The morphological phenotype induced by these activated *ras* genes was reproducibly dependent upon the particular mutation involved. Mutations which gave rise to gene products with the most potent transforming ability (measured by colony morphology) were those encoding valine, leucine, isoleucine, arginine and threonine at position 12. Cells expressing such gene products typically displayed a fully transformed morphology: the cells were consistently round and refractile, and they grew to the highest saturation densities. An intermediate class of mutants (serine, methionine, cysteine, tyrosine, phenylalanine, tryptophan, histidine, aspartate, glutamate, alanine and asparagine at position 12) generated transformants that overgrew the monolayer, although the cells displayed a less striking change in morphology. Mutants with the most subtle transforming activity (lysine and glutamine at position 12) induced foci only after extended times (typically 2-3 weeks). Such cells exhibited a nearly normal morphology. We failed to observe any focus formation in cells co-transfected with *ras* genes encoding either glycine or proline at position 12 throughout five separate experiments, each performed in duplicate. Figure 3 shows representative morphologies of G418-resistant Rat-1 cells co-transfected with these four classes of mutants.

To provide an additional measure of the transformed phenotype, we determined whether cultures co-transfected with each mutant *ras* vector could grow in an anchorage-independent

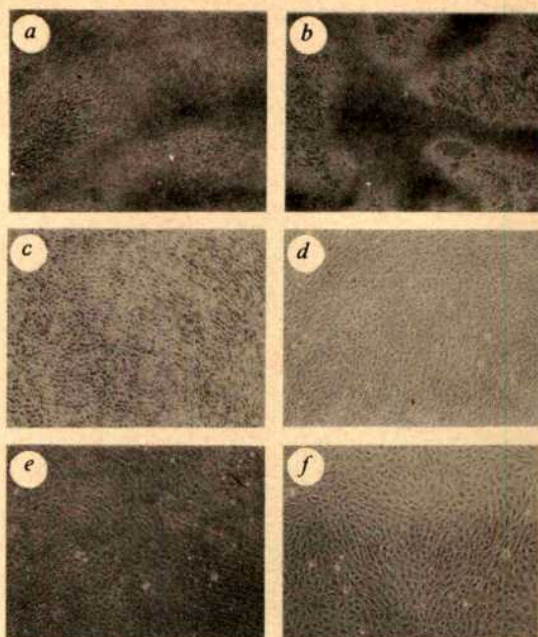


Fig. 3 Phase-contrast photomicrographs of Rat-1 cells ($\times 40$). Cells were transfected by *c-Ha-ras1* genes encoding substitutions at codon 12 of: a, valine; b, isoleucine; c, tyrosine; d, lysine; e, proline; f, untransfected Rat-1 cells.

Methods: Transfections were performed by a modification of the calcium phosphate precipitation method⁴⁹ on 1.0×10^6 Rat-1 cells using 5 μ g of each of the *c-Ha-ras1*-containing plasmids and 500 ng of pSVNeoBal6, a plasmid containing the neomycin-resistance^{32,33} gene isolated from Tn5 (ref. 33) under transcriptional control of the simian virus 40 early promoter (a 348-bp *PvuII*-*HindIII* fragment⁵⁰). After 48 h, the transfected cells were split 1:3 into selective medium containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Hyclone) and 400 μ g ml⁻¹ G418. After 14 days, resistant colonies were cloned using cloning cylinders, or alternatively on occasion passed *en masse*. Monolayers derived from individual clones or mass cultures were scored for morphology and photographs taken 8-16 days after the cells reached confluency. Foci which overgrew the monolayers were scored for their morphological phenotype (indicated by + to +++ on Table 1) as described in the text.

fashion. We found that all G418-resistant cell lines capable of forming foci on monolayers grew in soft agar (Table 1) with plating efficiencies of 0.2-6.0% (data not shown). Colonies co-transfected with Gly 12 and Pro 12 versions of *c-Ha-ras1* genes were unable to grow in agar, even when mass cultures derived from many (10-30) G418-resistant colonies were plated. Despite the additional selection for anchorage-independent growth following G418 selection, cells which were originally flat (those expressing Lys 12 or Gln 12 versions of p21) did not display any obvious morphological changes when replated following passage through agar (data not shown).

These results indicate that the presence of either a glycine or proline residue at position 12 of p21 results in a polypeptide incapable of inducing the morphological transformation of Rat-1 cells, at least when expressed under the transcriptional control of its own promoter. To verify that these vectors indeed directed the synthesis of p21, as well as to determine the relative levels of expression of p21, cloned cells from cultures co-transfected with representative mutant *ras* genes were examined for the expression of the *c-Ha-ras1* p21 gene product. Cellular proteins were labelled with ³⁵S-methionine and extracts were immunoprecipitated with rabbit immune sera raised against the *c-Ha-ras1* gene product expressed in *Escherichia coli*³⁶. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. Figure 4a illustrates that anti-p21 antiserum detects the *c-Ha-ras1* gene product in G418-resistant colonies co-selected with active transforming

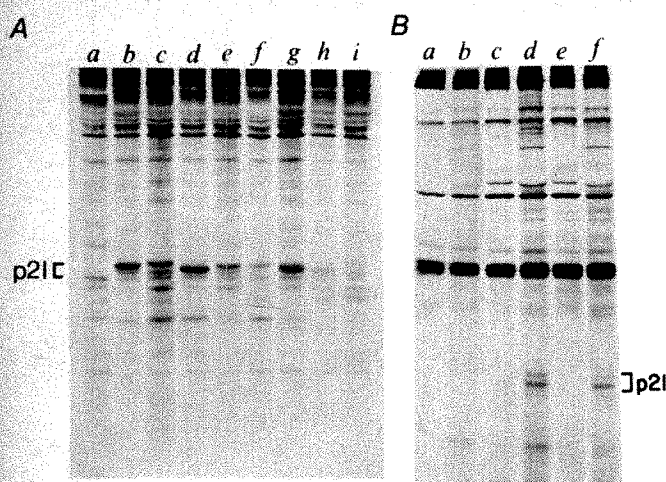


Fig. 4 Expression of p21 in transfected cell lines. **A**, ^{35}S -methionine-labelled cell lines were immunoprecipitated with rabbit anti-p21 serum (lanes b-i) or with preimmune serum (lane a). Cells were transfected by c-Ha-ras1 genes substituted at position 12 by: lanes a, b, valine; lane c, isoleucine; lane d, threonine; lane e, tyrosine; lane f, phenylalanine; lane g, arginine; lane h, lysine; lane i, untransfected Rat-1 cells. **B**, lysates were immunoprecipitated by anti-p21 rabbit serum (lanes b, d, f) or with preimmune rabbit serum (lanes a, c, e). Lanes a, b, untransfected Rat-1 cells; lanes c, d, cells were transfected by pPBLASph (glycine); lanes e, f, cells were transfected by the Pro 12 mutant.

Methods: Cultures were labelled with $150\ \mu\text{Ci}\ ^{35}\text{S}$ -methionine (Amersham) per ml methionine-free media for 3 h. Cells were lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.1% SDS, and 0.5% Aprotinin (Sigma). Lysates were preincubated with formaldehyde-fixed *Staphylococcus aureus* (Pansorbin, Calbiochem) for 30 min, and the bacteria were removed by centrifugation. The supernatants were immunoprecipitated with the anti-p21 polyclonal rabbit serum³⁶ or preimmune serum for 1 h at 4°C. Immune complexes were precipitated by *S. aureus* and the bacteria were washed three times with the lysis buffer. Immunoprecipitated proteins were analysed by electrophoresis on 15% or 12.5% SDS-polyacrylamide gels⁵² and visualized by fluorography after treatment with Enhance (NEN).

genes. Notably, each *ras* polypeptide exhibits a slightly different electrophoretic mobility, extending previous descriptions of p21 polypeptides which display altered electrophoretic mobilities as a consequence of single amino acid substitutions³⁷⁻³⁹. Human c-Ha-ras1 p21 was also detected in cell lines co-transfected with those c-Ha-ras1 genes unable to effect the morphological transformation of Rat-1 cells (Gly 12 and Pro 12) (Fig. 4b). In these cases, the p21(Gly 12) and p21(Pro 12) exhibit indistinguishable electrophoretic mobilities, as found for these polypeptides directly expressed in *E. coli* (W.W.C., unpublished results). These results verify that the failure of these two genes to transform Rat-1 cells was not due to unanticipated mutations preventing the expression of p21.

The observation that p21 *ras* can induce a panoply of morphological phenotypes in Rat-1 cells depending on the amino acid at position 12 is unexpected. Interestingly, certain replacements which engender p21 with a more restricted transformation potential *in vitro* (for example, Lys 12 and Ser 12) appear in retroviral cognates of transduced *ras* genes (v-bas (ref. 17) and v-Ki-ras (ref. 12) respectively). We suspect this underscores the contribution of quantitative effects as well as qualitative changes in p21 on transformation potential. Consistent with this, we have found that sequences extending 1.7 kb 3' of the *Sph*I site in the c-Ha-ras1 gene (containing the reiterated copies of a 28 base pair (bp) element¹⁹) facilitate the expression of p21; when the expression of p21 (Lys 12) is augmented about fivefold in this manner, a more fully transformed phenotype is induced (W.W.C., unpublished results). These findings, together with the observation that extreme overexpression of even normal *ras* genes can induce the morphological transformation of NIH 3T3

cells³⁰, imply that a threshold level of expression exists for each position 12 *ras* mutant, above which any may transform such cells, although this remains to be established for p21(Pro 12).

Computer analysis of the secondary structure of p21 led to the suggestion that the amino-terminal domain of the polypeptide consists of two helical segments separated by a coil region surrounding amino acid 12 (ref. 22). It was predicted that, as substitution of any other amino acid for glycine at position 12 would probably extend the flanking helical regions²², any mutation affecting the coding properties of codon 12 would activate the oncogenic potential of p21 (refs 22, 40). We have tested this prediction and confirmed that while most amino acid substitutions can activate the polypeptide, those least compatible with an α -helical structure (glycine and proline⁴¹) encode polypeptides incapable of morphologically transforming Rat-1 cells, at least when expressed under the control of the normal *ras* promoter. We note, however, that beyond the marked tendency of glycine and proline residues to break α -helical regions within proteins, residues with the highest reported helical potential⁴¹ do not correlate in any obvious way with those at position 12 of p21 which induce the most pronounced changes in cellular morphology. Note that even some amino acids with strong transforming potential (isoleucine, threonine, leucine) have yet to be identified as activating lesions in tumours. Although it is possible that this reflects an inability of some of the 'activated' genes to participate in the oncogenic pathway by which *ras* genes exert their *in vivo* influence, it is most easily explained by the fact that such changes require the substitution of at least two of the three nucleotides which comprise codon 12, whereas all activated genes characterized to date vary from the cellular proto-oncogene by a single nucleotide. If indeed all but two amino acids at position 12 can activate p21 by *in vivo* criteria, it is possible that those with a potentially limited *in vitro* transforming capability escape easy detection when assayed by usual transfection methods; if this is so, the frequency of activated *ras* genes detected to date in tumours may in fact be an underestimate.

The functional alterations directly responsible for the oncogenic activation of human *ras* proteins are unclear. Attempts to discern differences between normal and activated *ras* polypeptides with regard to specificity or extent of nucleotide binding^{36,42,43}, acylation, or subcellular localization⁴⁴ have been unsuccessful. The observation that p21 possesses an inherent GTPase activity which is selectively impaired by an activating Val 12 mutation³⁶ raises the possibility that an inability to hydrolyse GTP may constitutively activate the polypeptide. The availability of *ras* mutants such as those described here and elsewhere³⁹ which encode polypeptides of varying transformation potentials should facilitate further efforts to define the biochemical changes critical for oncogenic activation of p21.

We thank Dr Charles Benton for the preparation of anti-p21 antisera and Joel Hayflick for assistance with the mutagenesis.

Received 21 June; accepted 21 August 1984.

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Growth-rate dependent regulation of mRNA stability in *Escherichia coli*

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The rate of production of bacterial gene products is known to vary with the rate of cell growth, the concentrations of many cellular proteins are altered during times of decreased growth rate¹. In addition, proteins whose *in vivo* levels show no significant alterations with changes in cell doubling time must be synthesized at rates that vary in direct proportion to the growth rate of the cell. In certain instances, growth-rate dependent gene regulation has been shown to occur at the transcriptional or translational level^{2,3}. Another potentially important element in the regulation of gene expression is the stability of messenger RNA. We report here the effect of bacterial growth rate on the half lives of four different monocistronic *Escherichia coli* mRNA species. The stabilities of two of these species, the transcripts of the *ompA* and *cat* genes⁴⁻⁶, exhibit a marked dependence on cell growth rate, whereas the half lives of the transcripts of the *lpp* and *bla* genes^{7,8} are constant over a broad range of cell doubling times. Our results indicate that *E. coli* can alter the rate of synthesis of certain proteins by modulating mRNA stability in response to changes in the rate of cell growth.

The *ompA* gene of *E. coli*, which encodes a highly abundant outer membrane protein, specifies an mRNA transcript that has an unusually long half life^{4,5,9}. Previously, it has been shown that the amount of OmpA protein in the outer membrane remains constant throughout a wide range of bacterial doubling times¹⁰. Such invariability requires a mechanism for adjusting the rate of biosynthesis of this protein in direct proportion to the rate of cell growth. To determine whether this bioregulatory mechanism involves changes in the stability of the *ompA* transcript, we studied the decay of *ompA* mRNA in bacteria growing in conditions that yield a range of doubling times.

E. coli strain C600 was cultured at 30 °C in one of three bacterial growth media: L-broth, MOPS/glucose or MOPS/ace-

Table 1 Half lives of individual *E. coli* transcripts at different growth rates

Transcript half lives in bacteria grown in various media				
	mRNA segment length (nucleotides)	L-broth (40 min)	MOPS/glucose (100 min)	MOPS/acetate (200 min)
<i>bla</i>	587	3.3 ± 0.4	3.2 ± 1.0	2.4 ± 0.7
	267	3.1 ± 0.1	3.2 ± 0.5	3.1 ± 0.3
	80	2.8 ± 0.9	2.5 ± 0.5	2.5 ± 0.1
<i>cat</i>		2.3 ± 0.6	—	0.4*
<i>lpp</i>		20 ± 1	—	23 ± 3
<i>ompA</i>	598	13 ± 3	6.8 ± 0.8	4.4 ± 0.6
	340	15 ± 2	5.0 ± 0.3	4.4 ± 0.4
	200	25 ± 2	10.6 ± 2.7	3.4 ± 0.6
	85	21 ± 4	5.9 ± 0.4	3.8 ± 1.0

Half lives are reported in minutes. Messenger RNA segment boundaries for the *ompA* and *bla* transcripts are as previously defined⁹. The cleavage of a single phosphodiester linkage within a segment of a transcript is scored as the degradation of that segment. All experiments were performed with *E. coli* strain C600 growing exponentially at 30 °C. **Methods:** Decay data for the *ompA* and *cat* transcripts were obtained as described in Figs 1 and 2. For the analysis of *ompA* mRNA decay in cells multiplying with a doubling time of 100 min, RNA was extracted from *E. coli* growing in MOPS/glucose¹ 0, 4, 8 and 16 min after rifampicin addition. The decay of the *lpp* transcript in bacteria growing in L-broth and MOPS/acetate was examined by RNA blotting⁹. The RNA samples were collected 0, 20, 40 and 60 min after rifampicin addition, fractionated by gel electrophoresis, blotted onto diazophenylthioether paper¹⁶, and probed with nick-translated plasmid pKEN113, which contains a copy of the *E. coli lpp* gene¹⁷. Segmental analysis of the degradation of *bla* mRNA in *E. coli* growing in L-broth, MOPS/glucose and MOPS/acetate was performed as previously described⁹. Half lives were determined by densitometric analysis of autoradiograms. Rates of decay were calculated by least-squares determination of the slope of a plot of -ln (band intensity) against time. Errors were estimated from the standard deviation of the slope. For the *bla* transcripts, the half lives of only three of the seven characteristic segments⁹ are reported.

ate¹; in these media, the cells doubled every 40, 100 or 200 min respectively. The decay of *ompA* mRNA, and of its component segments, after the blockage of transcription by rifampicin was analysed by S₁ nuclease protection as described previously⁹. Figure 1 and Table 1 show that the half life of the *ompA* transcript, as judged from the stability of its least stable segment, fell from about 15 min in cells cultivated in L-broth (doubling time, 40 min) to about 4 min in cells cultivated in MOPS/acetate (doubling time, 200 min). Nutritional conditions that led to an intermediate growth rate (MOPS/glucose; doubling time, 100 min) resulted in an intermediate stability for the *ompA* transcript. The retardation of decay reported previously for the segments of the *ompA* transcript nearest the 5' end⁹ was not observed at the slow growth rate. In MOPS/acetate, all segments of the transcript showed approximately the same rate of decay (Table 1). This degradation pattern exhibited by the *ompA* transcript in bacteria grown under conditions that lead to an extended doubling time presumably results from a failure of the cells to accumulate decay intermediates, as observed previously for *bla* mRNA. We have proposed that such a decay pattern reflects rate-limiting initial cleavage of the full-length transcript⁹.

These measurements of mRNA half life rely on the fact that almost all (>95%) of the total RNA in *E. coli* is represented by stable RNA species: that is, ribosomal RNA and transfer RNA¹¹. Because these abundant and stable RNA molecules do not decay to any significant extent during the time course of an experiment for determining mRNA half life¹², they provide a convenient and reliable reference for measuring the rate of degradation of labile transcripts such as mRNA.

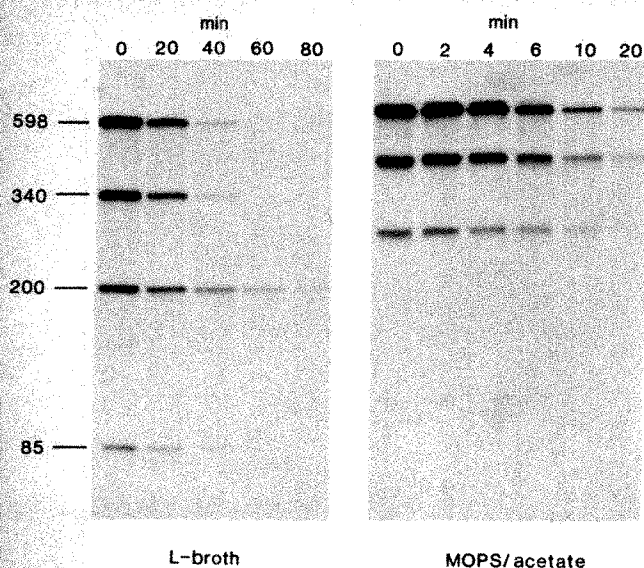


Fig. 1 Decay of *ompA* mRNA in *E. coli* cells growing with a doubling time of 40 min (left) or 200 min (right). Total cellular RNA from *E. coli* strain C600 growing exponentially at 30 °C in L-broth (doubling time, 40 min) was isolated 0, 20, 40, 60 and 80 min after rifampicin treatment⁹. Similarly, RNA from bacteria growing at 30 °C in MOPS/acetate¹ (doubling time, 200 min) was isolated 0, 2, 4, 6, 10 and 20 min after rifampicin addition. Segmental analysis of *ompA* mRNA decay was performed as previously described⁹. The bands correspond to the component segments of the *ompA* transcript; the order of the segments (5' to 3') is 200, 85, 598 and 340 nucleotides⁹.

In the absence of any compensatory effect on the initiation of transcription, a reduction in the stability of a transcript should result in a corresponding decrease in its intracellular concentration. Consistent with this prediction is our observation that the fraction of total cellular RNA represented by the *ompA* message falls by a factor of five in cells grown in MOPS/acetate as opposed to L-broth. We also find that the concentration of total cellular RNA, which consists primarily of the stable species rRNA and tRNA, drops by a factor of 1.8 when the bacterial doubling time increases from 40 min (cells grown in L-broth) to 200 min (cells grown in MOPS/acetate), in agreement with previous studies¹¹. Thus, we calculate that the steady-state concentration of *ompA* mRNA is reduced roughly by a factor of nine in bacterial cells growing in MOPS/acetate.

Because the concentration of OmpA protein in cells cultured under different growth conditions is constant¹⁰, the rate of synthesis of the OmpA protein must decrease by about a factor of five as the cell doubling time is increased from 40 to 200 min. The decreased rate of synthesis of OmpA protein that accompanies the prolonged cell doubling time in MOPS/acetate can be accounted for entirely by the observed change in *ompA* mRNA concentration; moreover, most of the reduction in the concentration of the *ompA* transcript can be attributed to its decreased stability.

To determine whether the observed shortening of the *ompA* mRNA half life in cells growing in MOPS/acetate medium is the result of slowed growth rate *per se*, or whether the effect is otherwise related to the media used, bacteria were grown in L-broth until their doubling time began to decrease on entry into the stationary phase. At a stage of growth corresponding to a doubling time of ~200 min, the cells were treated with rifampicin, and RNA was extracted at time intervals thereafter. Under these growth conditions, the half life of the *ompA* transcript was about 4 min, the same half life observed for *ompA* transcripts from cells having a 200-min doubling time resulting from growth in MOPS/acetate medium.

Like *ompA* mRNA, the *lpp* transcript encodes an abundant outer membrane protein (lipoprotein) and has the potential to form extensive secondary structure⁷. However, growth-rate

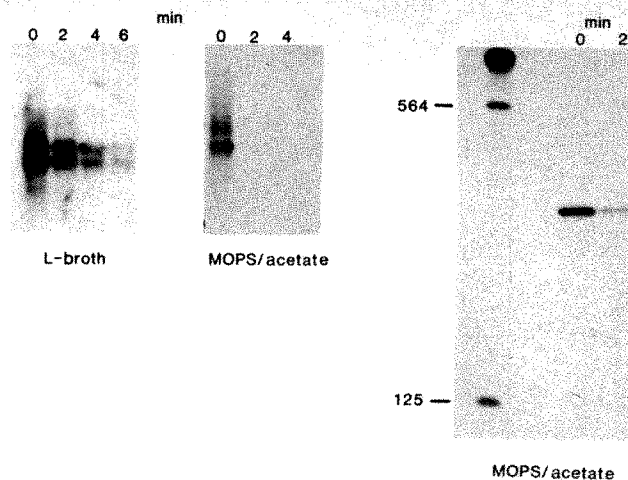


Fig. 2 Decay of *cat* mRNA in *E. coli* strain C600 containing the plasmid pACYC184 was grown to log phase at 30 °C in L-broth (left: doubling time, 40 min) or in MOPS/acetate (middle and right: doubling time, 200 min), and total cellular RNA was isolated 0, 2, 4 and 6 min after rifampicin treatment⁹. Analysis of *cat* mRNA decay was performed by fractionation of the RNA examples on a 1.2% agarose/formaldehyde gel²² followed by blotting onto a nitrocellulose filter²³ (left and middle). The blots were probed with nick-translated *cat* gene DNA. The decay of *cat* mRNA from *E. coli* growing in MOPS/acetate was also monitored by S₁ nuclease analysis²⁴ (right). Plasmid pACYC184 linearized with *Eco*RI and 5' end-labelled with [γ -³²P]ATP and polynucleotide kinase was used as the probe. The observed protected DNA fragment corresponds to the 5' terminal 270 nucleotides of *cat* mRNA²⁵. The finding of a unique 5' end by S₁ nuclease mapping demonstrates that the two *cat* messages detected by RNA blotting result from multiple sites of transcriptional termination.

dependent alteration of mRNA half life was not observed for the transcript of the *lpp* gene, which had a half life of ~22 min at 30 °C whether the bacteria were grown in L-broth or MOPS/acetate (Table 1). In addition, there was no change in the fraction of total RNA represented by the *lpp* transcript under these different growth conditions. Thus, an increased rate of mRNA decay with the slowing of cell growth is not a universal phenomenon, nor does it seem to be a general property of long-lived *E. coli* transcripts. Although it is not known whether the biosynthesis of the *lpp* gene product is stringently or loosely related to the rate of bacterial growth, our data demonstrate that any down-regulation of the rate of production of lipoprotein is not controlled at the level of mRNA stability.

The effect of growth rate on the stability of two *E. coli* mRNA species having the short half life more typical of *E. coli* messages was also examined. These were the *bla* transcripts of plasmid pBR322, which encode the periplasmic protein β -lactamase, and the *cat* transcripts of plasmid pACYC184, which encode the cytoplasmic protein chloramphenicol acetyltransferase (CAT)^{6,8,13-15}.

There are six overlapping *bla* messages transcribed from pBR322 (ref. 9). These transcripts are initiated at one of two promoters and are terminated at one of three sites. All six *bla* transcripts had a half life of 3 min independent of the growth medium used. Moreover, under all growth conditions, each of the component segments of *bla* mRNA⁹ decayed at the same rate (Table 1). When *bla* transcripts from cells isolated just before entry into the stationary phase of growth were analysed as described above for the *ompA* transcript, the half life was again found to be 2-3 min. As in the case of the *lpp* transcript, the fraction of total RNA represented by *bla* mRNA was the same at all growth rates, indicating that the steady-state concentration of *bla* mRNA fell by the same factor of 1.8 as did the overall RNA concentration in bacteria growing in MOPS/acetate. (The small decrease in *bla* mRNA concentration presumably reflects a slightly reduced rate of transcription.)

In contrast, the intracellular concentration of *cat* mRNA fell by a factor of ~13 when the cell doubling time was increased

Table 2 Enzyme activities of β -lactamase and chloramphenicol acetyltransferase from *E. coli* grown in different media

	L-broth	MOPS/acetate
β -Lactamase	13	43
CAT	0.37	0.25

Enzyme activities are units per mg of cellular protein. All experiments were performed with *E. coli* strain C600 containing either pBR322 or pACYC184. Different growth rates were achieved by growing cells in L-broth or in MOPS/acetate. Bacteria growing exponentially were ruptured by sonication, and the cell debris was removed by centrifugation. The supernatant was assayed for either β -lactamase activity or chloramphenicol acetyltransferase (CAT) activity^{18,19}. Total protein concentration was determined using the method of Lowry²⁰. The copy number²¹ of pACYC184 at the two growth rates was found to be the same, whereas the copy number of pBR322 increased twofold in MOPS/acetate.

from 40 to 200 min. Most of the reduction in *cat* mRNA concentration can be explained by the fact that the multiple transcripts of the *cat* gene, which result from the use of one promoter and two transcriptional terminators (Fig. 2), decayed approximately five times faster in response to the fivefold prolongation of doubling time. The *cat* messages isolated from bacteria cultivated in L-broth showed a half life of 2 min, whereas *cat* mRNA from cells grown in MOPS/acetate medium showed a half life of about 0.4 min (Table 1, Fig. 2).

Analysis of the activities of β -lactamase and CAT in cells growing in L-broth or MOPS/acetate shows that the concentrations of both enzymes, relative to total cellular protein, are altered under conditions of prolonged doubling time. In cells grown in MOPS/acetate, the β -lactamase concentration increases more than threefold, whereas the cellular concentration of CAT falls to two-thirds of its value in L-broth (Table 2). Bacteria grow in MOPS/acetate at one-fifth the rate seen in L-broth; by multiplying these factors, we calculate that the rate of synthesis of β -lactamase must be nearly independent of growth rate, whereas the synthesis of CAT must slow by a factor of ~ 8 in MOPS/acetate. As the steady-state concentration of *bla* mRNA is hardly influenced by growth rate (see above), it is not surprising that the rate of production of β -lactamase is similarly unaffected. In contrast, the substantial decline in the rate of synthesis of CAT in cells growing in MOPS/acetate can be accounted for by the observed decrease in the cellular concentration of *cat* mRNA, which is seen here to result primarily from a decrease in the half life of the *cat* transcripts.

The results reported here indicate that the decay rates of various messages are affected differently by changes in the rate of cell growth. Growth-related effects on decay occur with both a very stable mRNA species (*ompA*) and an mRNA species (*cat*) that has a half life of 2–3 min, as is typical of most *E. coli* mRNAs. The effect of growth on mRNA decay does not appear to be correlated with either the extent of mRNA secondary structure or the nature of the gene product. The *ompA* and *lpp* transcripts both possess the potential for extensive intramolecular basepairing, and both encode abundant outer membrane proteins; yet the decay of only one of these messages (*ompA*) is modulated in response to changes in the bacterial growth rate.

Our observations indicate that control of the rate of mRNA decay is one means by which cells can reduce the rate of biosynthesis of proteins during times of slow growth. Moreover, the finding that only certain *E. coli* messages are degraded more rapidly at slow bacterial growth rates implies the existence of mechanisms that differentially alter the decay rates of transcripts.

We thank Staffan Normark and Ulf Henning for stimulating discussions. These studies were supported by the Riksforskningsnämnden mot Cancer of Sweden and the US NIH.

Received 2 July; accepted 2 August 1984.

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Synthesis in yeast of a functional oxidation-resistant mutant of human α_1 -antitrypsin

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Cumulative damage to lung tissue by leukocyte elastase is thought to be responsible for the development of pulmonary emphysema, an irreversible lung disease characterized by loss of lung elasticity^{1–3}. It is also thought to be involved in the rapidly developing and usually fatal adult respiratory distress syndrome⁴. The primary defence against elastase damage is the anti-protease known as α_1 -antitrypsin^{5,6}, a glycosylated serum protein of 394 amino acids³. Oxidation of the methionine 358 residue located at the active centre of α_1 -antitrypsin results in a dramatic decrease in inhibitory activity towards elastase which effectively inactivates the protective function⁶. It has been suggested that this oxidation sensitivity has a regulatory function and allows tissue breakdown at sites of inflammation by inactivation of α_1 -antitrypsin by oxygen radicals released by phagocytes³. In the above diseases, however, the oxidative inactivation of α_1 -antitrypsin is probably of major importance in allowing lung damage by elastase^{4,7}. An oxidation-resistant α_1 -antitrypsin might make it possible to reduce the large doses of α_1 -antitrypsin required for emphysemics and provide treatment for acute inflammatory respiratory conditions. To further the possibility of therapy for the above conditions, we describe here the synthesis in yeast of active, non-glycosylated, human α_1 -antitrypsin. Site-directed mutagenesis has been used to construct an active, oxidation-resistant derivative containing a single methionine to valine substitution at the active centre. This demonstrates the potential of engineered modifications to protein molecules designed to improve their physiological function.

To obtain α_1 -antitrypsin cDNA sequences encompassing the entire coding region, a cDNA library constructed from poly(A) mRNA from liver was screened with radiolabelled oligonucleotide probes corresponding to amino acid residues –23 to –17 (25,000 colonies) and 344–350 (5,000 colonies). The probe sequences were taken from the partial nucleotide sequences described previously^{8,9}. Four colonies hybridized^{10,11} to the N-terminal probe and 148 to the C-terminal probe. After gel

Fig. 1 Nucleotide sequence and predicted amino acid sequence of α_1 -antitrypsin cDNA encoding the pre-protein. For expression in yeast the pre-sequence was removed by *Bam*HI digestion. The reactive centre Met-Ser at positions 358-359 is boxed. Amino acids above the sequence identify differences between this protein sequence and those derived from *a*, protein sequencing³; *b*, the cDNA of Woo *et al.* (see ref. 3); *c*, the cDNA of Bollen *et al.*²⁵.

Methods: Double-strand cDNA was synthesized as described previously³⁶, size fractionated on a Sepharose CL4B column to remove most of the cDNA of <300 bp, treated with *S*₁ nuclease and cloned at the *Pst*I site of a pBR322 derivative using the homopolymeric dG and dC tailing method³⁷. Note that the second-strand cDNA was self-primed; the first seven nucleotides of the cDNA differ from the genomic sequence and are probably incorrect as has been described previously³⁸. The following restriction fragments shown were isolated by preparative polyacrylamide gel electrophoresis³⁷: 603 bp *Bam*HI-*Bst*E2 from p5'AT-1; 450 bp *Bst*E2-*Ava*I from p3'AT-11; 85 bp *Ava*I-*Hin*II from p3'AT-3. The five C-terminal amino acids and termination codon were encoded by the synthetic oligonucleotide adapters shown adjacent to the coding sequence. These oligomers were synthesized by phosphoramidite chemistry²⁸, phosphorylated³⁷, and mixed in equimolar amounts. Approximately 2 pmol of fragments 1 and 2 were ligated³⁷ together in 50 μ l and after removal of ligase, digested with *Bam*HI and *Ava*I. Similarly, fragment 3 and the synthetic duplex were ligated and digested with *Ava*I and *Sall*. The resulting fragment mixtures were ligated, digested with *Bam*HI and *Sall* and DNA of ~1,200 bp gel isolated and cloned by substitution into *Bam*HI, and *Sall* cut and phosphatased³⁷ pBR322. Analysis of miniscreen plasmid DNA³⁷ from these transformants indicated that a full length cDNA had been obtained and one such transformant was used in subsequent constructions.

-24		-20	-10	
GGGGGGGGGGAGGGTAATGACAA		ATG CCG TCT TCT TCG TGG GGC ATC CTC CTG CTG GCA AGC GTG TGC TGC CTG GTC CCT GTC TCC CTG GCT		
		Met Pro Ser Ser Val Ser Trp Gly Ile Leu Leu Ala Gly Leu Cys Cys Leu Val Pro Val Ser Leu Ala		
Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr Ser His His Asp Gln Asp His Pro Thr Phe Asn Lys Ile Thr Pro Asn Leu				30
GAG GAT CCC CAG GAG GAT GCT GCC CAG AAG ACA GAT ACA TCC CAC CAT GAT CAG GAT CAG CCA ACC TTC AAC AAG ATC ACC CCC AAC CTG				90
<i>Bam</i> HI				
		40	50	60
Ala Glu Phe Ala Phe Ser Leu Tyr Arg Gln Leu Ala His Gln Ser Asn Ser Thr Asn Ile Phe Phe Ser Pro Val Ser Ile Ala Thr Ala				180
GCT GAG TTC GCC TTC Asp CTA TAC CCG CAG CTG GCA CAG TCC AAC AGC ACC AAT ACT TTC TCC CCA GTG AGC ATC GCT ACA GCC				
Phe Ala Met Leu Ser Leu Gly Thr Lys Ala Asp Thr His Asp Gly Ile Leu Gly Gly Leu Asn Phe Asn Leu Thr Gly Ile Pro Gly Ala				90
TTT GCA ATG CTC TCC CTG GGG ACC AAG GCT GAC ACT CAG GAT GAA ATC CTG GAG GGC CTG AAT TTC AAG CTC AGG GAG ATT CCG GAG GCT				360
		100 Arg(b,c)	110	120
Gln Ile His Gly Gly Phe Gln Gly Leu Leu His Thr Leu Asn Gln Pro Asp Ser Gln Leu Gln Leu Thr Thr Asp Gly(c)				450
CAG ATC CTG GAA GGC TTC CAG GAA CTC CTC CAT ACT CCG CAC AAC CAG CCA GAC AGC CAG CTG CAG CTG ACC ACC GGC AAT GGC CTG TTC CTC				
Ser Gly Gly Leu Lys Leu Val Asp Lys Phe Leu Gly Asp Val Lys Lys Leu Tyr His Ser Gly Ala Phe Thr Val Asn Phe Gly Asp Thr				540
AGC GAG GGC CTG AAG CTA GTG GAT AAG TTT TTG GAG GAT GAT GAT AAG TTG TAC CAG TCA GAA GCC TTC ACT GTC AAC TTC GGC GAC ACC				
Glu Glu Ala Lys Lys Gln Ile Asn Asp Thr Val Gly Lys Gly Thr Gln Gly Lys Ile Val Asp Leu Val Lys Gly Leu Asp Arg Asp Thr				630
GAA GAG GCC AAG AAA CAG ATC AAC GAT TAC CTG GAG AAG GGT ACT CAA GGG AAA ATT GTG GAT TTG GTC AAG GAG CTT GAC AGA GAC ACA				
Val Phe Ala Leu Val Asn Thr Ile Phe Phe Lys Gly Lys Trp Glu Arg Pro Phe Gly Val Lys Asp Thr Gly Glu Glu Asp Phe His Val				720
GTT TTT GCT CTG GTG AAT TAC ATC TTC TTT AAA GGC AAA TGG GAG AGA GCC TTT GAA GTC AAG GAC ACC GAG GAA GAG GAC TTC CAC GTG				
Ala(b)		220	230	240
Asp Gln Val Thr Thr Val Lys Val Pro Met Met Lys Arg Leu Gly Met Phe Asn Ile Gln His Cys Lys Lys Leu Ser Ser Trp Val Leu				810
GAC CAG CTG ACC ACC GTG AAG GTG CCT ATG ATG AAG CGT TTA GGC ATG TTT AAC ATC CAG CAC TGT AAG AAG CTG TCC ACC TGG GTG CTG				
<i>Bst</i> EII				
		250	260	270
Leu Met Lys Tyr Leu Gly Asn Ala Thr Ala Ile Phe Phe Leu Pro Asp Glu Gly Lys Leu Gln His Leu Glu Asn Glu Leu Thr His Asp				900
CTG ATG AAA TAC CTG GGC AAT GCC ACC GCC ATC TTC TTC CTC CTT GAT GAG GGG AAA CTA CAG CAG CTG GAA AAT GAA CTC ACC CAG GAT				
Ile Ile Thr Lys Phe Leu Glu Asn Glu Asp Arg Arg Ser Ala Ser Leu His Leu Pro Lys Leu Ser Ile Thr Gly Thr Tyr Asp Leu Lys				1,170
ATC ATC ACC AAG TTC CTG GAA AAT GAA GAG AAG TGT GCC AGC TTA CAT TTA CCC AAA CTG TCC ATT ACT GGA ACC TAT GAT CTG AAG				
<i>Eco</i> RV				
		310	320	330
Ser Ile Leu Gly Gln Leu Gly Ile Thr Lys Val Phe Ser Asn Gly Ala Asp Leu Ser Gly Val Thr Gly Glu Ala Pro Leu Lys Leu Ser				1,260
AAG ATC CTG GGT CAA CTG GGC ATC ACT AAG GTC TTC AGC AAT GGG GCT GAC CTC TCC GGG GTC ACA GAG GAG GCA CCC GTG AAG CTC TCC				
Lys Ala Val His Lys Ala Val Leu Thr Ile Asp Gly Lys Gly Thr Gly Ala Ala Gly Ala Met Phe Leu Glu Ala Ile Pro Met Ser				1,350
AAG GCC GTG CAT AAG GCT GTG CTG ACC ATC GAG GAG AAA GGG ACT GAA GCT GCT GGG GGC ATG TTT TTA GAG GCC ATA CCC				
Pro Pro Glu Val Lys Phe Asn Lys Pro Phe Val Phe Leu Met Ile Glu Gln Asn Thr Lys Ser Pro Leu Phe Met Gly Lys Val Val Asn				590
CCC CCC GAG GTC AAG TTC AAC AAA CCC TTC GTC TTC TTA ATG ATT GAA CAA AAT ACC AAG TCT CCC CTC TTC ATG GGA AAA GTG GTG AAT				
<i>Ava</i> I				
		594		
Pro Thr Gln Lys OC				
CCC ACC CAA AAA TAA DTGGCTCTGGCTCTCTCAAGCCCTCCCTCCATCCGTGGCCGCTCCCTGGATGACATTAAGAAGGGTGTGAGCTGGAAAAA				
<i>Hin</i> II				
CCC ACC CAA AAA TAG				
GGG TGG GTT TTT ATC AGCT				
<i>Sall</i>				

analysis of plasmid DNA from the 4 N-terminal and 12 C-terminal clones, three overlapping clones were selected for further study. We have elucidated a composite DNA sequence for α_1 -antitrypsin derived from the three cDNA plasmids by subcloning and sequencing in M13 phage vectors^{12,13} (Fig. 1). The protein sequence predicted by the cDNA differs from the published sequences in several positions, probably due to the genetic polymorphisms known to be quite common in α_1 -antitrypsin¹⁴.

To assemble a cDNA encoding the mature α_1 -antitrypsin suitable for expression in yeast, three restriction fragments from the *Bam*HI-*Hin*II sites shown in Fig. 1 were first isolated from their respective plasmids. These fragments were ligated together with the synthetic DNA segment shown in Fig. 1 and cloned by substitution between the *Bam*HI and *Sall* sites of pBR322¹⁵.

To construct a yeast plasmid directing the synthesis of α_1 -antitrypsin we first isolated the yeast acid phosphatase (PHO5) promoter from a library of yeast genomic DNA using a synthetic oligonucleotide probe (Fig. 2). The PHO5 promoter was chosen because it is a relatively strong yeast promoter which can be regulated using either phosphate concentration¹⁶ or temperature-inducible mutants¹⁷. The second codon of the acid phosphatase gene was joined in phase to the second codon of the α_1 -antitrypsin cDNA by blunt-ended ligation (Fig. 2). After subcloning, this PHO5- α_1 -antitrypsin segment was isolated as a *Bam*HI-*Sall* fragment and joined to a *Sall*-*Bam*HI fragment containing the transcription terminator of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (ref. 18 and our unpublished results), followed by insertion into the pC1/1 yeast shuttle vector as a *Bam*HI fragment.

To change the methionine residue at position 358 to a valine residue, we used M13 site-directed mutagenesis¹⁹. The *Pvu*II-

Sall fragment of the cDNA (Fig. 1) coding for amino acids 238-394 was cloned by *Sma*I-*Sall* substitution into M13 mp10¹³. Single-stranded viral DNA was isolated and used as a template for site-directed mutagenesis using a synthetic 30-mer as a mutagenic primer (Fig. 3). Positive plaques were identified using the primer as a probe and the codon change was confirmed by DNA sequencing (Fig. 3). The 360 base pair (bp) *Eco*RV-*Sall* fragment of α_1 -antitrypsin (Fig. 1) containing the mutation, was inserted into the yeast expression plasmid as described in Fig. 3 legend. This fragment was re-sequenced from pC1/1PHO5AT(Val), confirming the T to C transition and showing that no other mutations had occurred during the M13 mutagenesis, as have been observed previously²⁰. Yeast strain AB103²¹ was transformed²² with plasmids pC1/1, pC1/1PHO5AT(Met) and pC1/1PHO5AT(Val).

To characterize the α_1 -antitrypsin produced in yeast, cultures were grown in high (repressing) or low (inducing) phosphate and collected in log phase. Cells were lysed with glass beads, and the soluble cell proteins analysed by SDS-polyacrylamide gel electrophoresis and elastase inhibition assays (Fig. 4; Table 1). The results of the gel analysis (Fig. 4A) show that a protein of ~42,000 molecular weight (MW) is specifically induced in those transformants containing the α_1 -antitrypsin cDNA. A protein of 44,000 MW is expected for mature, non-glycosylated α_1 -antitrypsin. Although strain AB103 contains the *pep*4-3 mutation and is deficient in most of the proteolytic activities of the yeast vacuole²³, additional smaller proteins of ~23,000 and 19,000 MW were specifically induced. These were shown to be antigenically related to α_1 -antitrypsin by Western blot analysis (Fig. 4B) and possibly result from proteolytic degradation. When synthesis of α_1 -antitrypsin was similarly analysed in strain

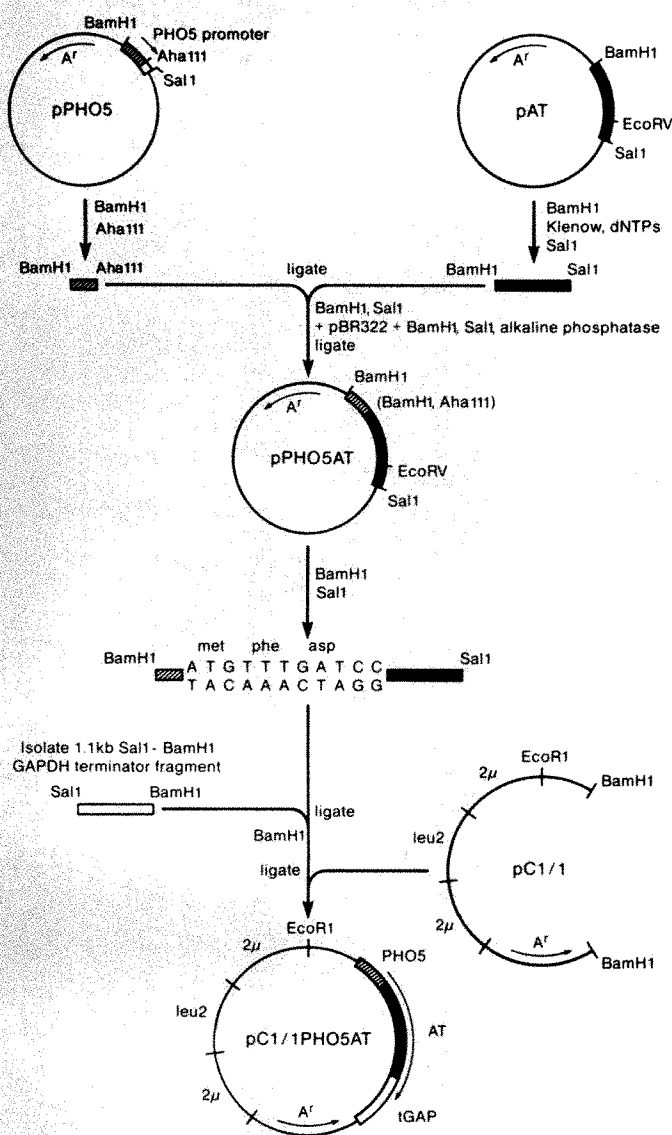


Fig. 2 Construction of yeast plasmids expressing a PHO5- α_1 -antitrypsin fusion encoding the first two amino acids of the PHO5 gene and residues 2-394 of α_1 -antitrypsin. DNA fragments and plasmids are only approximately to scale. To isolate the PHO5 promoter, the oligonucleotide probe ^{5} GGCACT-CACACGTGGGACTAG ^{3} , complementary to a portion of the sequence of the promoter, was used to screen a library of *Saccharomyces cerevisiae* DNA³⁹. Several positive clones were obtained and one was sequenced^{12,13} and shown to contain the PHO5 promoter. A 630 bp *Bam*HI-*Sal*I fragment from this isolate was subcloned into pBR322. This fragment contains 80 bp of the acid phosphatase coding sequence and has the sequence ^{5} ATGTTTAAA ^{3} encoding the first three amino acids, the second and third codon specifying an *Aha*III site. pC1/1 is a derivative of pJDB219 (ref. 40) in which the PMB9 sequences have been replaced by pBR322.

2150 *pep4*⁺, an increased level of the 23,000 and 19,000 MW fragments was observed as well as the production of a new fragment of 40,000 MW (data not shown).

To determine the activities of α_1 -antitrypsin(Met) and α_1 -antitrypsin(Val), appropriate yeast extracts were incubated with human leukocyte elastase and a synthetic peptide substrate whose cleavage can be assayed by increase in absorbance at 410 nm⁶. Inhibition of elastase was found to be directly proportional to the amount of human α_1 -antitrypsin added in the presence or absence of control yeast extracts (Table 1 assays 1-5, 13, and our unpublished results). Control yeast extracts had no effect on elastase activity (Table 1, assay 6) whereas

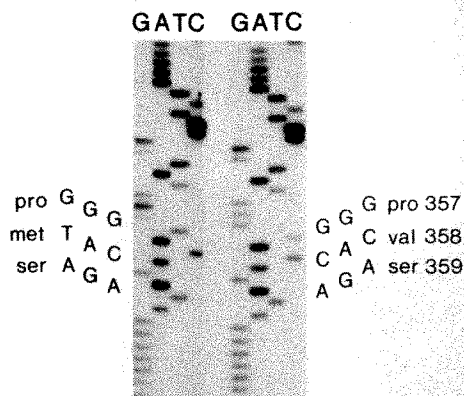


Fig. 3 Autoradiogram of the DNA sequencing gel showing the nucleotide sequence of wild-type (Met) and mutant (Val) active centres of α_1 -antitrypsin.

Methods: A mutagenic primer having the sequence ^{5} GACCTCGGGGGGATAGACACGGGTATGGC ^{3} was synthesized and used to prime second-strand DNA synthesis on single-stranded M13 containing a wild-type α_1 -antitrypsin fragment (see text) as described previously^{19,21}. The mismatch is underlined. Mutagenized phage were located using the same primer as a probe and filters were washed for 60 min at 65 °C in 0.15 M NaCl, 15 mM sodium citrate (1×SSC). To substitute the Met to Val mutant in the yeast expression vector shown in Fig. 2, the 360 bp *Eco*RV-*Sal*I fragment of α_1 -antitrypsin (Fig. 1), containing the mutation, was prepared from a digest of the M13 replicative form DNA. This was ligated to the 1,350 bp *Bam*HI-*Eco*RV fragment, containing the PHO5 promoter and N-terminal residues of α_1 -antitrypsin, prepared from pPHO5AT, and the 1,100 bp *Sal*I-*Bam*HI GAPDH terminator fragment, both shown in Fig. 2. After ligation, the mixture was digested with *Bam*HI before ligation into *Bam*HI-cut and phosphatased pC1/1. After transformation of HB101⁴¹, one positive clone, analogous to pPHO5AT(Met), was obtained after restriction enzyme analysis of plasmid DNA from 12 clones.

Table 1 Effects of *N*-chlorosuccinimide oxidation on α_1 -antitrypsin activity in yeast extracts assayed by inhibition of elastase activity

Assay no.	Plasmid	Volume of yeast extract (μl)	μg α_1 -Antitrypsin	NCS (μl) (10 mM)	Elastase activity (%)
1	—	—	—	—	100
2	—	—	0.1	—	85
3	—	—	0.2	—	67
4	—	—	0.5	—	22
5	—	—	1.0	—	0
6	C1/1	1.5	—	—	100
7	Met	0.5	—	—	54
8	Met	1.5	—	—	8
9	Val	0.5	—	—	52
10	Val	1.5	—	—	15
11	—	—	1.0	—	5
12	—	—	1.0	10	91
13	C1/1	3.8	1.0	—	12
14	C1/1	3.8	1.0	10	84
15	Met	1.5	—	—	8
16	Met	1.5	—	10	93
17	Val	1.5	—	—	20
18	Val	1.5	—	10	18

Yeast extracts were prepared by lysis with glass beads and centrifuged to remove cellular debris³². Extracts were diluted in 50 mM Tris pH 8, 50 mM NaCl. For *N*-chlorosuccinimide (NCS) oxidations, 7.5 μl of extracts were incubated at room temperature for 5 min in 100 μl of 50 mM Tris pH 8, with the indicated amount of NCS (10 mM). Aliquots were transferred to tubes containing 0.1 mM Meo-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (Vega), 50 mM Tris pH 8, 0.5 M NaCl, 100 μg bovine serum albumin (BSA), in a volume of 1 ml. Assays were initiated by the addition of 0.15 μg HLE and incubated for 15 min at 28 °C, terminated by the addition of 100 μl of 8 M acetic acid and the absorbance at 410 nm was determined⁶. Protein concentrations³³ were: C1/1, 6.4 mg ml⁻¹; Met, 8.0 mg ml⁻¹; Val, 9.7 mg ml⁻¹. From the amount of human leukocyte elastase (HLE) activity observed, given the protein concentration of the yeast extracts and the amount of HLE added, the percentage of the yeast-soluble protein that is α_1 -antitrypsin can be calculated. We assume 100 % HLE activity, a 1:1 complex between HLE and α_1 -antitrypsin, and that the molecular weights of HLE³⁴ and α_1 -antitrypsin are 29,000 and 42,000 MW, respectively. In this experiment α_1 -antitrypsin is 3.0 and 3.5 % in the Met extract (assays 7 and 8) and 2.3 and 2.6 % in the Val extract (assays 9 and 10).

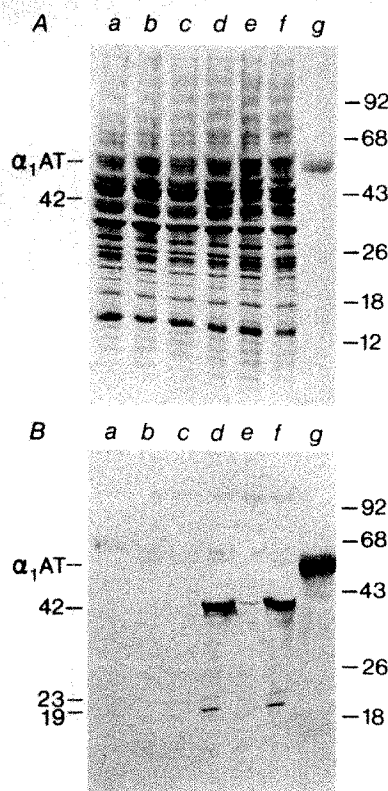


Fig. 4 α_1 -antitrypsin (α_1 AT) and related proteins synthesized in yeast. **A**, SDS 10–20% polyacrylamide gradient gel⁴² stained with Coomassie blue showing soluble proteins synthesized in: *a*, uninduced pC1/1-containing control cells; *b*, induced pC1/1-containing control cells; *c*, uninduced pC1/1PHO5(Met) cells; *d*, induced pC1/1PHO5(Met) cells; *e*, uninduced pC1/1PHO5(Val) cells; *f*, induced pC1/1PHO5(Val) cells; *g*, glycosylated α_1 -antitrypsin from human serum. **B**, Proteins from gel **A** which react specifically with antibody to α_1 -antitrypsin. Identical extracts were electroblotted on the same gel, transferred to nitrocellulose paper and α_1 -antitrypsin-related proteins visualized by antibody-conjugated horseradish peroxidase staining⁴³. Elastase inhibition assays (Table 1) indicated that induced extracts contained 1.0 % α_1 -antitrypsin in this experiment.

Methods: Yeast cells were grown in 50 ml cultures of leu⁻ selective media in high or low phosphate to an A_{650} of ~2. After lysis³², ~20 μ g of the soluble proteins were electroblotted on a 10–20 % SDS-polyacrylamide gradient gel and blotted onto nitrocellulose paper. α_1 -Antitrypsin-related proteins were visualized by washing the nitrocellulose paper with rabbit anti- α_1 -antitrypsin (Accurate Chemicals) followed by horseradish peroxidase/goat anti-rabbit antibody staining⁴³. Media containing high and low levels of phosphate consisted of synthetic complete medium lacking leucine⁴⁴ supplemented with 0.2 volumes of phosphate-depleted YEP medium⁴⁵, 2 g l⁻¹ asparagine replacing (NH₄)₂SO₄, 1 g l⁻¹ KH₂PO₄ (high phosphate) or 1 g l⁻¹ KCl, 0.03 g l⁻¹ KH₂PO₄ (low phosphate). Yeast strains are AB103.1 (*Mata leu2-3,112 ura3-52 pep4-3 his4-580* [cir^o]) and 2150-2-3 (*Mata leu2 ade1* [cir^o]).

induced yeast extracts for α_1 -antitrypsin(Met) and α_1 -antitrypsin(Val) both inhibited elastase activity (Table 1, assays 7–10). The percentage of α_1 -antitrypsin in yeast extracts (Table 1) showed approximate agreement with estimates from SDS-polyacrylamide gels, indicating normal activity of the recombinant α_1 -antitrypsins.

To discover if α_1 -antitrypsin(Val) was oxidation resistant, we treated extracts with *N*-chlorosuccinimide, which converts susceptible methionines to their sulfoxides²⁴. Both the recombinant α_1 -antitrypsin(Met) and a reconstituted control extract containing human α_1 -antitrypsin are susceptible to oxidative inactivation whereas the α_1 -antitrypsin(Val) is resistant (Table 1, assays 13–18).

It has been reported recently that the development of emphysema can be limited by intravenous injection of α_1 -antitrypsin partially purified from human serum²⁵. Although this enzyme is the most abundant anti-protease in human serum (~1.3 g l⁻¹)²⁶, insufficient amounts are available from plasma to treat all affected individuals because its half life is only 6 days²⁷. Production from yeast cells offers a viable alternative, especially as yields of >10 % of soluble cell protein as α_1 -antitrypsin have been obtained recently using the glyceraldehyde phosphate dehydrogenase promoter²⁸ (manuscript in preparation). It will, however, be important to establish whether the half lives in serum and the rates of inhibition are comparable for the non-glycosylated recombinant α_1 -antitrypsins. The replacement of methionine by valine at the active centre of the molecule was based on the observation that a synthetic peptide with this substitution is an improved substrate for elastase²⁹. Recent studies have shown that a naturally occurring arginine substitution at this position lacks elastase inhibitory activity but has gained the specificity of antithrombin III³⁰. Thus, it is important to determine the specificities of any derivatives with therapeutic potential. In addition to providing improved treatment for pathogenic deficiencies of α_1 -antitrypsin, the construction of novel α_1 -antitrypsin derivatives by protein engineering, together with kinetic⁶ and crystallographic³¹ studies, should provide a better understanding of the regulation and reaction mechanism of this important anti-protease.

We thank James Travis for gifts of α_1 -antitrypsin and HLE, James Travis and Robin Carrell for useful discussions, Robert Huber for ref. 31, Qui Lim Choo, Kathy Mead, Bernardita Mendez, Patricia Tekamp-Olson, and other colleagues at Chiron for help with experiments and the manuscript.

Received 10 May; accepted 30 August 1984.

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Giving 'em the business

W.F. Clocksin

The AI Business: Commercial Uses of Artificial Intelligence.

Edited by Patrick H. Winston and Karen A. Prendergast.

MIT Press: 1984. Pp.324. \$15.95, £14.25.

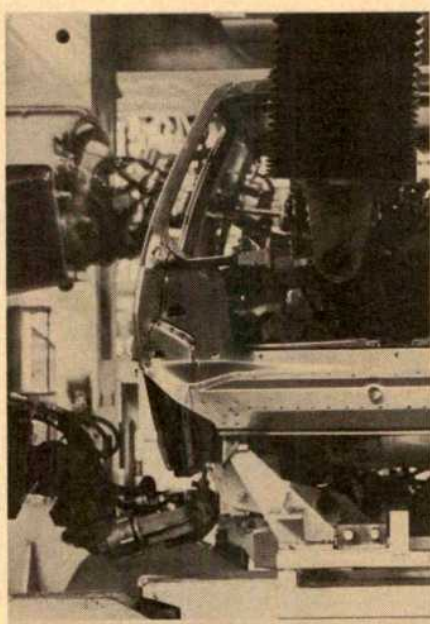
THE past, it seems, is not always another country. In the 1960s, over-optimistic advocacy by researchers of the nascent field of artificial intelligence (AI) created expectations impossible to satisfy. In the 1980s, spurred by the prospect of serious international competition, we again have enthusiastic promotion of the technology of AI — especially expert systems and “fifth-generation” computers — and it is no less fervent: but this time the promoters move in commercial circles. The passionate propaganda from the marketing men dismays the researcher and perplexes the technical manager; as in the 1960s, the hyperbole obscures a realistic assessment of the true potential of AI technology.

In an attempt to clear the air, a colloquium was called by the MIT Industrial Liaison Program to bring together representatives of the four main groups involved: the academics, who started it all; the investment bankers and venture capitalists; those concerned with industrial research and development; and those in industry who use techniques devised by AI researchers because there is a job to be done. *The AI Business* is a collection of the edited transcripts of the colloquium. The book opens and closes with perspective and summary chapters by the editors. The first chapter sets the scene by placing MIT at the centre of AI research, and by giving a short “history” of the field complete with its own Prehistory, Dawn Age, Renaissance and so forth. The practising researcher may find it difficult to take this chapter seriously, while others may be dangerously misled by such cavalier treatment. Similarly, while the last chapter does a fine job of summing up, and offers some homespun advice on what action should be taken by universities and companies, there is again the tendency to lapse into dottiness, viz., “...strength in Artificial Intelligence ensures strength at everything that Artificial Intelligence can help with. And that may be just about everything”. These two chapters are very much the bread of the sandwich though; the meat is proved by the 16 other contributors.

Although Winston (and later, Minsky) admits to regarding the term “expert system” with a certain amount of scorn, the casual reader will not understand why, for the first section of the book contains six chapters describing some of the best examples of work in the field. Here the practitioner will find accounts of his old friends XCON and DIPMETER

ADVISOR, true products of AI research which have reached commercial credibility not by hype, but by quietly saving their users substantial amounts of money.

The next section, entitled “Work and Play”, contains six chapters on a subject close to the hearts of AI researchers: interactive workstations. Part of AI



Robots on the line — automatic welding on the Robogate system at Fiat's Rivalta plant in Italy.

research is about making computers easier to use, particularly as automated design assistants and as apprentices. This goes much farther than the computer-aided draughting found in industry today. The computer should be a participant in the design process, synthesizing candidate designs that conform to sets of constraints with which the designer experiments. There is a fascinating contribution by Alan Kay, who rambles through a compelling mixture of reminiscence and advice. Other chapters cover commercially available systems for electronic design automation and English-language communications with databases. The overall theme of this section is the life-cycle from basic research to exploitation in the marketplace.

The next section deals with robotics. Mike Brady gives a good survey of the relationship of AI research areas to their application in industrial robotics, while the other two contributions survey real-life industrial applications, giving examples of sensor-controlled robots and more general

issues in factory automation. This is the most straightforward and informative section of the book, perhaps as a result of the authors' application of well-understood techniques to well-defined problems, with clear criteria for the success or failure of an application. Other areas of AI are not yet as much under control.

The final section, “Today and Tomorrow”, centres on trends, both technological and economic. Minsky's chapter is a prolonged snort of disapproval at contemporary events in research, politics, business and industry. His basic message, though, is of prime importance. The commercial successes of today rely on AI research carried out ten years ago. Because there is practically no basic work in AI now being done, the future may already be lost. This theme is repeated in the other two contributions by investment analysts. Particularly impressive is the cogent and analytical chapter by the economist, William Janeway. Anyone closely involved with recent Government initiatives in “information technology” in any country will find considerable sympathy with Janeway's view that,

The packaging of ignorance by artifice to obtain finance distorts not only the returns actually received by investors. Distortion of research activity in order to obtain finance on such terms is both a professional and a social cost that we in this country seem ill equipped to appreciate.

The main question to be asked of this book is, “Who is it for?”. As the editors point out in the last chapter, the agreements and differences of opinion expressed at the colloquium have been preserved in the book. The transcripts of the informal discussion sessions at the end of each section are particularly effective in this connection. Great stuff for the practising researcher, who will be intimately familiar with the elusive issues involved, and who will thus be in a position to read between the lines. However, the discussions, together with the editors' own contributions, give the book a tone of superficiality and off-handedness which is dangerous and inappropriate for a more general audience. In spite of this, some of the individual chapters are particularly sound, and deserve wider exposure.

I want to see technical managers and engineers becoming acquainted with the issues covered in this book. But I would tell them not to believe everything they read in it. The simplistic, shoot-from-the-hip flavour may be instinctively appealing, but it endangers credibility, and should not be interpreted as solid worldly-wise experience. Despite Winston's “Six Ages of Artificial Intelligence”, 20 years is not a very long time. What we need, above all, is a little patience. □

W. F. Clocksin is an Assistant Director of Research at the Computer Laboratory, University of Cambridge.

Ways with MCAs

Karol Sikora

Monoclonal Antibodies: Probes for the Study of Autoimmunity and Immunodeficiency.

Edited by Barton F. Haynes and George S. Eisenbarth.

Academic: 1984. Pp.318. \$45, £33.

Monoclonal Antibodies: Principles and Practice.

By James W. Goding.

Academic: 1984. Pp.276. \$22.50, £17.

THE immune system has evolved into one of nature's most perfect defences. Recognition, memory, specificity and a plethora of effector mechanisms all combine to create a powerful and dynamic defence network. But things can go wrong. In autoimmune disease the immune response turns its attention to host tissues and organs causing self-destruction. In immunodeficient states, whether congenital or acquired, the host becomes susceptible to a wide range of normally innocuous infectious agents as well as to an increased risk of malignancy. The problem in dissecting out the immune system's game plan is the multiplicity of players. Monoclonal antibodies (MCAs) have changed this. These highly specific molecular flags enable the precise identification of the system's components; the lymphocyte.

For the first time in one book, Barton Haynes and George Eisenbarth have brought together a collection of reviews on the use of MCAs in the analysis of immunological disorders. Beginning with a summary of the study of T-cell differentiation using the well-known anti-T-lymphocyte subset MCAs, the book proceeds through a series of accounts of haemopoiesis, thymic differentiation, human hybridoma formation and immunodeficiency. The final four chapters discuss classic endocrine diseases such as diabetes, thyroid problems and myasthenia gravis.

The net result is a rather hotchpotch collection. To the immunologist there is little new that has not been reviewed elsewhere, often by the same authors. The clinician searching for a clearing in the increasingly complex immunological jungle will also be disappointed; haematologists, rheumatologists and endocrinologists will not find much of relevance here to their current practices

More on monoclonals

• *Monoclonal Antibodies to Receptors: Probes for Receptor Structure and Function*, edited by M.F. Greaves. Publisher is Chapman & Hall, price is £35, \$75.

• *Monoclonal Antibodies and Functional Cell Lines: Progress and Applications*, edited by R.H. Kennett, K.B. Bechtol and T.J. McKearn, a sequel to an earlier volume of 1982. Publisher is Plenum, price is \$59.40, £47.03.

and how to improve them. Too much data is presented in many chapters instead of the critical reviews which would have been far more helpful. An exception is the chapter on diabetes, which succinctly covers the role of the immune system in the causation of the disease. The editors might consider using this contribution as a model for an improved future edition.

I can be a great deal more enthusiastic about the second book reviewed here, that by James Goding. Although MCAs were discovered nearly a decade ago, there are few practical guides to the experimenter setting up a monoclonal factory. Goding is a scientist of considerable experience in the MCA business, and has written such a guide, a good one.

After a chapter on the theory behind MCA production, the remainder of the book goes step by step through the various stages of production, purification, radiolabelling, the application of MCAs in assays and in immunohistology also being discussed. Particularly pleasing is the step-wise layout of the various practical

procedures described, together with the extensive reference material provided. Of course, no two workers use precisely the same approach as outlined but for the newcomer to the field the recipes given do provide a starting point.

Surprisingly little information, however, is given to guide the neophyte in choosing which myeloma system to use, often a major stumbling block. Several are mentioned but no comparison between them is given. And there is no discussion of the more recently developed forms of immunization using peptide fragments; by preparing MCAs to peptides synthesized on data predicted from DNA analysis, the circle between gene and protein can now be closed.

Altogether, though, this is an excellent laboratory manual, invaluable to the newcomer to the field. But new editions will be required frequently if it is to remain useful over the next few years. □

Karol Sikora is Director of the Ludwig Institute for Cancer Research, Cambridge, UK.

Climate of quality

J.M. Walker

Climates of the Oceans. World Survey of Climatology, Vol. 15.

Edited by H. van Loon.

Elsevier: 1984. Pp. 716. \$173, Dfl. 450.

FEW books can have had as difficult a gestation period as *Climates of the Oceans*. The first editor died, the second withdrew, and when Harry van Loon took over, in 1974, 11 years after the book was commissioned, only one chapter was available in a form suitable for publication. New authors had to be found for four of the other six chapters and, furthermore, Dr van Loon had to accept the constraints imposed by the editorial decisions of his predecessors. The publishers must have wondered if the book was jinxed. Fortunately, they persevered.

The result is an academic textbook and work of reference worthy of its place in the *World Survey of Climatology* series. Moreover, the book has admirably filled a gap in the literature; until now there has been no authoritative and comprehensive survey of weather and climate over the oceans. For that reason it will find a wide audience, including not only meteorologists, oceanographers and geographers, but also those who need information about marine weather and climate for commercial purposes.

As is almost inevitable in a book of this kind, the chapters vary in length, quality and style, but the overall standard of writing and presentation is high. Paradoxically, given the paucity of observational data from most oceanic regions south of the Equator, the longest

chapters are those dealing with the oceans of the Southern Hemisphere. O Höflich's contribution on the climate of the South Atlantic occupies 191 pages; N.A. Streten and J.W. Zillman's (South Pacific) 167; and J.J. Taljaard and H. van Loon's (Indian Ocean south of 35°S) 97. In contrast, M.A. Einarsson's chapter on the climate of Iceland takes up only 25 pages (and the reader is left to guess why it is included in a book on ocean climates!).

It is regrettable that the book is not as up to date as it might have been. It contains few references to works published since 1980. Indeed in "Climate of the Indian Ocean North of 35°S" by C.S. Ramage, the most recent reference is dated 1975, and in K. Terada and M. Hanzawa's chapter on the North Pacific it is 1974. The obvious conclusion is that contributions were submitted over a period of years and were not subsequently updated. This is a pity, because in the past decade there have been numerous studies of atmospheric behaviour over the oceans, particularly over the North Atlantic, the North Pacific and monsoonal parts of the Indian Ocean. In Ramage's article, for example, there is no mention of the Monsoon Experiment (MONEX), while G.B. Tucker and R.G. Barry (who deal with the North Atlantic) say very little about the GARP Atlantic Tropical Experiment (GATE).

All things considered, however, it is remarkable that the shortcomings of the book are so few. It is a work of high quality and a mine of information and data. For anyone with an interest in the oceans it will be invaluable. □

J.M. Walker is Senior Lecturer in Meteorology and Physical Oceanography at the University of Wales Institute of Science and Technology, Cardiff.

Husbanding resources

Colin P. Groves

Evolution of Domesticated Animals.

Edited by I.L. Mason.

Longman: 1984. Pp.452. £35.

As archaeozoology has evolved into a discipline in its own right, so there has been a surge of interest in the economics of domestic animals and in the possibility of domesticating new species. Until recently there was little in the way of synthesis on the subject, and no general books had appeared since Zeuner's heavily archaeologically orientated *A History of Domesticated Animals* (Hutchinson, 1963). Now, suddenly, there is a rush to fill the vacuum. Two recent books of note have been Juliet Clutton-Brock's *Domesticated Animals from Early Times* (Heinemann, 1981), a general book suitable as an undergraduate text, and Helmut Hemmer's *Domestikation: Verarmung der Merkwelt* (Vieweg, 1983), so far not translated into English but an essential read for anyone interested in the background to and consequences of the domestication process itself.

Now comes this compilation, intended, as the introduction says, as

a single-volume account of the origin and history of domestic animals, species by species, which will not only be a ready source of exact information but will also provide an introduction to the literature.

In other words, it is not a theory-orientated book like Hemmer's, nor a textbook like Clutton-Brock's, but a source book of data and literature.

There are 69 chapters on different species or groups, including some on potential domesticates and some on experiments in progress. The longer chapters are divided into standard headings: introduction (with sections on nomenclature, general biology, and numbers and distribution); wild ancestor; domestication and early history; recent history and present status; and future prospects. The treatment of these various themes is very variable, and reflects the main interests of the authors of the chapters. Bökönyi is an archaeologist; nearly half of his chapter on the horse is taken up by the section "Domestication and Early History". Epstein and Bichard are both specialists in animal husbandry; more than half of their chapter on the pig is "Recent History and Present Status". Many other contributions are similarly weighted; perhaps the most successful and rounded of them are those by co-authors with different specialities (such as the chapter by Novoa, a veterinarian, and Wheeler, an archaeologist, on llama and alpaca), or by single authors who have made one or a group of species their own (the chapter on the dog, by Clutton-Brock;

on the reindeer, by Skjenneberg; and the series on domestic fowl, turkey and goose, by Crawford). There is always at least a nod towards all the statutory topics, though in some instances an author may be way out of his depth: Ryder flounders desperately amid the complexities of the taxonomy of wild sheep, and is not always happy with archaeological concepts (it is surprising to see his apparent unquestioning acceptance of high proportions of juveniles as evidence for domestication in an archaeological context, a line of argument which one would have thought long since thrown into disrepute), but as soon as he starts writing about wool, his lifelong speciality, he is clear, authoritative and a joy to read.

The section on general biology is, one would have thought, pivotal to each chapter, but perhaps because it is part of the introduction it is in most instances disappointingly skimmed over. Some of the best accounts of species' biology are, unexpectedly, in the chapters on minor species (Bali cattle, by Rollinson; yak, by Bonnemai).

My other main criticism of the book is its lack of an overview. Charles Reed does provide an introductory chapter, "The Beginnings of Animal Domestication", but this is of course from an archaeological point of view, and one would have liked to see similar chapters to cover themes such as the biology of domestication; the question of parallel changes in different species as a consequence of domestication; the suitability of different species for domestication; prospects for new domestication; and so on. I may have misunderstood the purpose of the book here, but it seems to me that discussion of such generalities would have been helpful from both a theoretical and a practical standpoint.

When all is said and done, however, it is a pleasure to read experts expounding the mysteries of their art and the book remains a monumental success. It is a compilation of essential data — the only one there is. It deals with all species that have ever been domesticated, or conceivably ever could be: the African elephant is there, the European bison, the onager (though I personally think this ought not to have been included), the ostrich, the crocodile. We have the latest on the cane-rat domestication project from the University of Ibadan group; hamsters and gerbils; snails and oysters; and a fascinating survey of silkworms. Whatever its defects, the book will stand for years to come as an invaluable reference work. It will deservedly find a place in the libraries of universities, animal husbandry and veterinary institutes, archaeology teams, and even many schools. And thank you, Mr Mason, for doing it; because it needed to be done. □

Colin P. Groves is a Senior Lecturer in the Department of Prehistory and Anthropology, Australian National University, Canberra.

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Pollination renewed

Peter D. Moore

Pollination Biology. Edited by Leslie Real. Academic: 1984. Pp.338. Hbk \$49, £36; pbk \$19.50, £15.

"FIGS, yuccas and specialized orchids have proved to be the exception rather than the rule." Thus Peter Raven introduces this compendium of review papers in pollination biology as he seeks to emphasize that it is a subject in which great changes are taking place. The period of descriptive work, from which the extraordinary stories of host-specific fig wasps and yucca moths emerged, is now past and experimental research on population variation and the selective advantage gained by specific individuals with particular attributes has begun.

As in most areas of ecology, the direction in which pollination biology is heading is towards a search for generalities rather than specifics. Can one, for example, make general statements about the evolution of breeding systems in plants in relation to pollination mechanisms? Can one quantify the degree of competition between plants for pollinators? Or is it possible to assess the adaptive significance of a specific trait, such as flower colour? These are the sort of problems which the book addresses.

The approach is radical, in places almost iconoclastic. Many old and well-engrained dogmas are critically examined and discarded, though they are not always replaced with simple alternatives. Take the evolution of dioecy, for example. It has long been assumed that the separation of sexes on to different plants provided advantages in terms of out-breeding. Here Wyatt claims that this principle is not supported by experimental evidence, but what does account for the development of dioecy is still somewhat obscure. Perhaps it is a matter of resource allocation, sexual selection or predation avoidance.

A chapter by Stephenson and Bertin is devoted to sexual selection in plants and approaches the subject in a logical way by dividing it into two categories: (i) competition between members of one sex for reproductive access to the other sex and (ii) choice of a partner by one of the sexes. Too often these groups have been confused in botanical studies; it is not difficult to understand how males may compete for access to females, but less easy to visualize the element of female choice. The latter is perhaps best regarded as non-random fertilization following pollen deposition, that is, some form of incompatibility. But this is questionably comparable to mate selection in animals. There is also the possibility that the female can favour specific male plants or pollinators by such characteristics as a restriction of flowering time. An interesting outcome of these processes is that sexual selection in plants

has not led to the visually obvious secondary sexual characters found in animals, but may often have influenced non-visual, physiological and biochemical developments.

This is a book which tackles many topics of interest to geneticists, ecologists and palynologists. Under what circumstances is wind pollination advantageous? What sensory stimuli determine the foraging behaviour of a pollinating insect? How does the density of a population of flowering plants affect the frequency of visits from a pollinator? These are exciting questions which cut across the boundaries of traditional disciplines.

Viewed as an undergraduate text, which it claims to be in part, the book is not

entirely satisfactory. The text is laden with multiple citations; too many subjects are discussed from different angles in separate chapters; and summaries of chapters are often inadequate as a guide to chapter content. But as a source of information and, above all, stimulating ideas for specialists and research workers, this is an extremely valuable addition to a literature which is currently dominated by detail and description rather than a conceptual approach. Figs and yuccas may be the exceptions, but the mundane may yet prove to be even more enthralling. □

Peter D. Moore is Reader in Ecology in the Department of Plant Sciences, King's College, University of London.

Wholesome genetics

Nick Proudfoot

Eukaryotic Genes: Their Structure, Activity and Regulation.

Edited by N. Maclean, S.P. Gregory and R.A. Flavell

Butterworths: 1983. Pp.474. £52, \$99.95.

THE explosion in our understanding of the structure and function of eukaryotic genes is nearly matched by the explosion in books describing it. However, it would seem that Maclean, Gregory and Flavell have produced one of the best accounts yet of the subject.

Far from being a disparate collection of short articles in incompatible styles (as is so often the case), the different reviews contained within the book have been carefully stitched together, in relatively homogeneous and concise format. Another valuable feature is that most of the material is up to date, to 1983, though such praise obviously applies more to some chapters than others. Before each section (group of related reviews), an introduction or summary has been provided. Some of these attempts to be enthusiastic to the extent of light-heartedness; for example: a "collection of DNA segments... has been called either a library (for the intellectuals) or a bank (for the businessman)". I idly wonder which editors refer to gene libraries or gene banks!

My detailed estimation of different sections in the book is as follows. In Section I on gene regulation (which would have been better named "Structural Aspects of DNA in Chromatin"), the first review by Jean Thomas is, to my mind, excellent; it told me all I felt I needed to know about this somewhat biophysical end of the subject. The other two reviews here, dealing with non-histone proteins and DNA modification, are somewhat less useful, though this probably reflects the fact that we know rather little about the involvement of, for example, HMG proteins and DNA methylation in gene regulation.

Section II might again have been more appropriately entitled, this time "Organization of 'Unusual' Chromosomes in Eukaryotes" rather than the general title "Organization of Chromosomes". However, the reviews on mitochondrial, lampbrush and polytene chromosomes are very helpful — so many molecular biologists are familiar with various "classic" experiments carried out using these systems but don't really understand their biology.

The third section covers experimental approaches. I found it somewhat disappointing. Why no specific articles on *in vitro* transcription, transient expression and stable transformation of cloned genes? Indeed, the only chapter I felt enthusiastic about here was that discussing the oocyte-egg system. Not that a review of teratocarcinoma and its use in studying gene expression isn't welcome; it is just that more generally-used systems should have had priority.

Section IV on specific gene systems is clearly excellent; while the order of the reviews is again a bit strange, there is here a wealth of information hard to glean in total from other sources. The book ends with a pathetically (and regrettably) short section on plant genes, but its brevity is hardly the fault of the editors. Taking away yeast, which in any case is barely a plant or indeed a eukaryote, very little progress has been made. Perhaps this will encourage more of us to turn to plants. After all, as Goldberg says in his review, plant genes have all the familiar boxes in

TATA, Cap, GT, AG, AATAAA,

so that one could still feel a "mainline molecular biologist".

Both advanced students and more experienced researchers will benefit from reading this book. I suspect it will find a sizeable audience, large enough for the publishers seriously to consider bringing out a cheaper edition. □

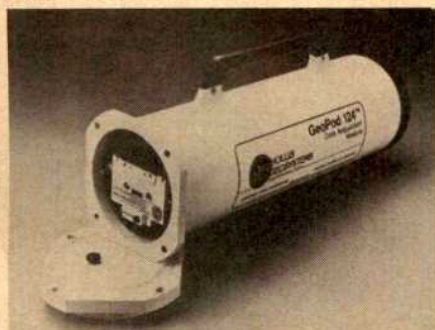
Nick Proudfoot is a University Lecturer and Tutor in Biochemistry at Brasenose College, University of Oxford.

From ecosystems to electrophoresis

New products covered this month include a completely sealed ecosystem being offered as a "gift item" and a computerized densitometer for electrophoresis.

● The use of linear roller bearing action in the new Shandon Hypercut rotary microtome is claimed to ensure a light, smooth cutting operation without sacrificing durability. This feature enables the operator to maintain a controlled cutting speed, producing sections of wax-embedded tissue blocks of an even, consistent thickness in a range from 0.5 to 30 μm . The Hypercut comes complete with a universal knife holder which features a unique lateral adjustment and accepts a wide range of knives, including glass, tungsten carbide tipped and disposable types.

Circle No. 100 on Reader Service Card.



For remote recording, the Hollis GeoPod.

● The GeoPod 124 data acquisition module is a long-term, magnetic tape cassette recording system designed for service in the field and in harsh industrial environments. Used in conjunction with its accompanying tape reader, the M-800, the GeoPod 124 is compatible with the IBM PC, DEC and other computing systems. Suggested uses for the GeoPod 124, from Hollis Geosystems, include meteorology, monitoring conditions on gas pipe-lines, hydrology, and structural strain measurements.

Circle No. 101 on Reader Service Card.

● New from Link Systems Ltd, the AN10000 microanalysis system is capable of measuring, storing, analysing, displaying and manipulating X-ray and electron signals produced by scanning and transmission electron microscopes. The basic 256K central processor is fast, flexible, and operates within a purpose-designed disk operating system which incorporates Winchester technology hard disks as a standard feature. AN10000 is equipped with a high resolution colour monitor, used in conjunction with a keyboard and MOUSE. The latter gives the operator a high degree of control and selection over displayed information.

Circle No. 102 on Reader Service Card.

● Simultaneous determination of nitrogen, carbon and sulphur is possible using two new versions of the Carlo Erba NA 1500 automatic nitrogen analyser. Samples of up to 100 mg (depending on sample composition) can be analysed. Other features are the short analysis times (3 min for N, 5 min for N and C, 6 min for S and 8 min for N, C and S), the wide measuring range (from 100 p.p.m. up to 100% for N, C and S) and high accuracy (better than 0.2%).

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● Quickly changed, non-breakable, chemically inert fluoroplastic capillaries are used as the disposable tips for the new Oxford pipettes. Called the Ultra Micro, this series of five pipettes is designed to provide relatively high reproducibility and accuracy at 1, 2, 3, 4 or 5 μl . Both top and bottom of the internal air displacement chamber are sealed by O rings, for leak-free operation. Fluoroplastic (FEP) capillary tips can be used for tasks such as stirring a sample into a cuvette without fear of tip breakage. And it only takes a few seconds to change tips.

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● The latest scanning electron microscope from the Electron Optics Group at Philips guarantees 5 nm resolution at 30 KV with 4 nm easily attainable. The SEM 515 attains higher resolution results from improved gun geometry, which gives a better signal-to-noise ratio. In a special low kv configuration this provides a fivefold increase in brightness when compared with the standard configuration. A new feature is the opportunity to include microprocessor-controlled automatic focusing and astigmatism correction. Based on algorithms developed by the Philips research laboratories in Hamburg, fully automatic focusing and astigmatism correction can be achieved in the most difficult circumstances.

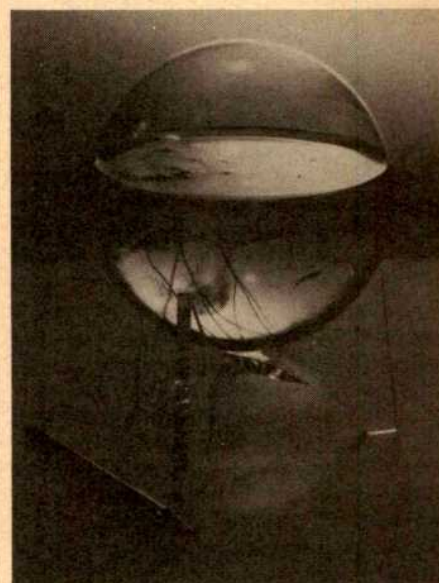
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● Malvern Instruments' new Zetasizer II is designed to measure the surface charge on particles suspended in liquid media and also to analyse their size distribution. The sizer makes use of high-speed digital correlation analysis of the light scattered by the particles as they move under the influence of an electric field. The charge on the particles is automatically computed and displayed. Particle size measurement — in the range 10 nm to 3 μm — is based on analysis of Brownian motion of the particles.

Circle No. 106 on Reader Service Card.

● A digital storage oscilloscope, data acquisition and waveform analysis system — the Computerscope — is being offered by Advanced Instrumentation & Measurement Systems Ltd (AIMS) of Oxford. The system upgrades a general purpose microcomputer for use in high-speed data acquisition and analysis. The Computerscope includes all hardware and software necessary to convert a common microcomputer into a multi-purpose instrument at a much lower cost than traditional "stand alone" instruments that incorporate their own pre-programmed microprocessor. There are two models. The HR14 has up to four channels with a maximum sampling rate of 500 kHz at 14-bit resolution (0.006%) and Model D2 has two channels and a maximum sampling rate of 3.5 MHz at 8-bit resolution (0.4%). The Computerscope can also operate as a spectrum analyser or signal averager.

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Shrimps and foliage share an EcoSphere.

● The glass globe above, little bigger than a softball, is an "EcoSphere". Engineering & Research Associates, Inc. (SEBRA) has taken a concept developed by space scientists and produced a "small-scale model of life on Earth". EcoSpheres are primarily intended as gifts but schools and museums have expressed interest in the EcoSphere as a teaching tool. The sphere contains a system including live shrimps, but the glass sphere is completely sealed, with no "breather" tube to supply exogenous oxygen.

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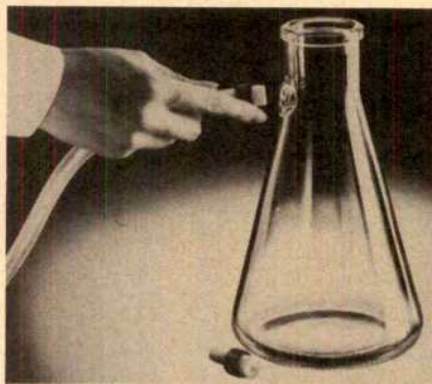
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What to do to help in India

Mrs Indira Gandhi's assassination is unlikely to see a retreat for support for Indian science. But in the turmoil, it may be neglected. People elsewhere should do more than they think possible to help.

THOSE who believe that the scientific community is in some sense international had better pay special attention to the plight in which Indian scientists will now find themselves. The assassination last week of Mrs Indira Gandhi will be, for them, more than a mere political calamity. The scientific enterprise in India, whatever its faults may be, owes its growth since independence from the British in 1947 to the policies deliberately followed first by Jawaharlal Nehru, the first Prime Minister of India, and then by his daughter. Their objectives were open and straightforward. Understanding the natural world would in due course be a source of economic prosperity, social improvement and the banishment of superstition.

It is ironical that the most striking proof so far of the wisdom of this policy, the new prosperity of the cereal-growing regions of northern India, may have helped to stir the fears of the fundamentalist Sikhs that their emotional hold on their followers would be attenuated, thus provoking the fortification of the Golden Temple at Amritsar, the Army's counter-move and last week's hail of bullets. There is no risk that Mrs Gandhi's son and her successor will draw the wrong lesson from this sad sequence of events, and conclude that things have been moving too quickly. The danger is, rather, that the new government will be so overwhelmed with the problems abounding in the wake of the assassination (not to mention the election soon to be held) that it will have neither the energy nor the resources for an enterprise whose benefits will be delayed beyond tomorrow.

That is why this is a time for recognizing once more the immediate social benefits to contemporary India of its scientific establishment. This huge country, which is in reality six or seven countries, suffers from a curious sense of lacking a cultural identity. Regional diversity is only part of the trouble. Mrs Gandhi may have been right in complaining (see page 93) that outsiders have no perception of how the long occupation by the British impoverished the country.

For at least a century, the intellectual life of India has been profoundly influenced by the universities, originally created on the pattern of the University of London in 1857. Over the years, the system has generated a distinguished roll of intellectual heroes, whose names are widely known and who are generally respected. The difficulty, for India, is that the achievements of these heroes are in a foreign idiom, one that may even be held to have been imposed from the outside. Part of the Nehru-Gandhi commitment to science and technology may be explained by the need to make a distinctive mark. That, certainly, is why centres of excellence are scattered through India. And the commitment had already gone so far, before independence, that the brief Janata government of the 1970s found it impractical to aim at what it foolishly supposed to be simpler goals. Having come so far, India cannot go back — which does not imply that chairmen of atomic energy commissions should not be sanskrit scholars on the side, or that the distinctive Indian sense of kinship (with places as well as people) need diminish.

The scientific enterprise also has much to offer in the solution of other problems rife in contemporary India. Since the time of Homi Bhabha, in the 1950s, the government of India has been dedicated to the belief that scientific enterprises would help to teach the country as a whole more productive ways of working. The Atomic Energy Commission was the spearhead of this move-

ment, and has now spawned a host of other institutions in the same or a similar mould — from the space programme to superb medium-sized laboratories such as the Physical Research Laboratory at Ahmadabad. The commission's plans for building nuclear power stations may have been less realistic than it had been hoping, but it has offered India a lesson now being widely learned — that difficult technical tasks can be carried through by people who are resolute enough, and meticulous, that it is not necessary in the process that each qualified person's hands should be held up by a small army of assistants and that it is possible for very large projects to be carried through without the corruption endemic in much of India's commercial life. The enterprise is a model of how to get good things done.

In principle, the scientific enterprise is also a model for the cure of that common if less corrosive social disease in India — the bureaucracy that seems to flourish everywhere. The difficulty is inescapable in a democracy where procedures derive from law, but need not be as much an impediment as it is in India. The difficulty has become a serious problem chiefly because those who operate the procedures derive power and therefore pleasure from the processing of pieces of paper that determine what other people should do. The scientific enterprise, however, recognizes that it cannot flourish when hamstrung in this way, and has in many places been able to make a dash for freedom. That is another model that the new government needs to have to hand.

Successive Indian governments' commitments to the goal of self-reliance (potential self-sufficiency) is more controversial. The late Lord Blackett found himself in hot water when he declared, when delivering the first Nehru Memorial Lecture in New Delhi, that it would be prudent for India to take fuller advantage of the willingness of people and governments elsewhere to help with the innovation then so plainly necessary. The shock is still remembered. There is something in the view, which Mrs Gandhi held, that a country as potentially powerful as India cannot perpetually be beholden to others. But may this not be a time when a softening of the line would be wise, if only to allow those who wish to help have something they can do?

The problem of the poor is different. Nobody denies the opinion general in India that a person is not less deserving of respect because he or she happens to be illiterate. But can such a person be as dispassionate, and as solid a member of society, as India's ambitions for democracy would require, if he is more likely to be hurt by infectious diseases or natural catastrophes? India may be lucky not have driven bulldozers through its villages in some rash attempt to make the country modern, but it is hard to believe that the enrichment of the poor would be calamitous. Ironically, there are many in the technical community of India anxious to do more, as Mrs Gandhi, to help with the rural poverty. Is this a time to catalyse their energies?

Governments swept into office in tragic circumstances such as those in India now are of two kinds — those who change nothing for fear that any change will bring more tragedy and those who recognize change to be a way of leavening a sense of shock by a sense of hope. Pandit Nehru and then Mrs Gandhi were persuaded that their scientific enterprise would indeed engender a better India. Their successor would be readily applauded if he were to ask for still greater efforts from those with whom rests the future of Indian agriculture, health and industry.

Outside India, there is also much that can be done. A critical account of science in India (*Nature* 308, 581;1984) provoked a stream of entertaining correspondence with a single theme — how much more Indian scientists (at home and overseas) might do to help India if they had a chance. May not this be a time for them to try a little harder? More to the point, may it not also be a time when the colleagues of Indian scientists, in India and elsewhere, should exert themselves more energetically to help? Nobody would pretend activities such as these would quell communal unrest, making it safe for Sikhs to walk the streets again. But both parts of the now divided community will need to know that their divisions will eventually be submerged in a wider unity. Go give a lecture, send a book, write a letter, is too trite for anything but a personal exhortation. Governments in the West can do more, and should, to help the enterprise along. An intelligent woman's death requires no less. □

Europe in the sky

Europe is preparing for adventures beyond the atmosphere, but without much thought.

ONCE upon a time (in the early 1960s) Great Britain was a disappointed space power (as the saying goes), with a design for a rocket launcher (called *Bluestreak*) whose development it could not afford to finish. So what more natural, Great Britain said to its European neighbours, than that everybody should pool resources and set up a common organization (eventually called *ELDO*, for European Launcher Development Organization) to rescue something from the frustrated enterprise? Everybody also agreed that space would be expensive, and so agreed that there should be a common effort on research (whence *ESRO*, for European Space Research Organization). After many adventures (and a great deal of poor management provoked by indecision), the governments of Western Europe decided that one organization would be enough (called *ESA*, for European Space Agency). But all this time, Great Britain knew it could get what it wanted more cheaply by making a deal with its friends across the Atlantic (or with *NASA*, for National Aeronautics and Space Administration, an agency of the US government). But then the time came when the US government was also short of money, wanting to spend every penny it could find on other kinds of rockets, so that it started asking its partners to dig in their pockets as well as its own. But poor Great Britain was by this time really poor and had nothing at all to spend. And so *NASA* flew off with its rich new friends — France, West Germany and Italy. Poor Great Britain had no choice but to set up a committee to ponder what to do.

This is how legend will encapsulate the developments last week, in Britain and on the mainland, in European policy on space. The cause of everybody's difficulty is *NASA*'s request for paying participants in the manned space platform. France, West Germany and Italy have it in mind to reply with a counter-proposal, one likely to satisfy *NASA*'s need to show to Congress that people elsewhere are prepared to help. Britain's trouble is not merely that the Science and Engineering Research Council (*SERC*) which would normally have an important say in such a project, is just now wondering which parts of basic science should be abandoned, but that the government as a whole is having to decide from which of two or three ministries to extract a further £1,000 million of spending money. The mainland, long since impatient, will be exasperated.

The moral in the tale is, however, simple. The Richmond committee (see page 92) has advised *SERC* to throw its lot in with *ESA* because its terms of reference were too narrow to allow a more adventurous proposal. What happens to British space policy in the next ten years is, however, beyond the competence of grant-making agencies. The government, not the council, should now decide what should be done, preferably in concert with its European partners, say what it intends to do and then stick with its decision. Making such a decision would no doubt be hard, but not making one will have even more serious consequences. □

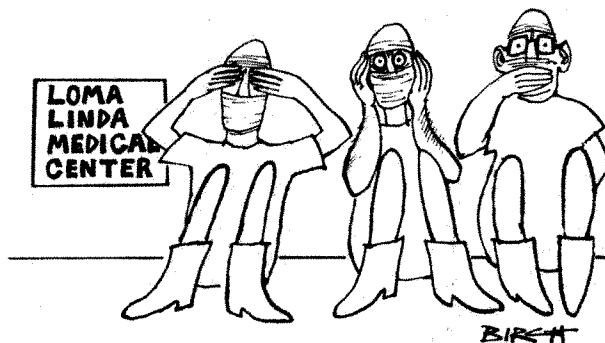
Grandstand medicine

Baboon's hearts are not taboo for people, but medicine which is not essential is wrong.

THE baboon-heart transplant performed on a critically ill infant at Loma Linda Medical Center in California is disquieting. As a medical milestone, to be sure, it is a remarkable accomplishment. Conceivably, it will someday even be a genuine treatment for the 1 in 3,000 infants born with hypoplastic left heart syndrome, the uniformly fatal defect from which the infant in California suffered. Moreover, it is important that the causes of disquiet should not be misrepresented. The need to sacrifice a healthy baboon so that a human infant might live is simply not the ethical dilemma that animal rightists, with an unfailing nose for the sensational, are pretending. A society that overwhelmingly approves of eating animals (other than people) would be even more astonishingly illogical than usual in its reaction to special cases if it were to deny the propriety of using a lower animal to save a human life. And the mere fact that an animal organ is being inserted in a human is an ethical issue only to those whose residue of mythological sentiment overpowers their capacity for rational thought; is there strictly a difference between the use of pig insulin and baboon hearts to make good deficiencies in human beings?

The serious difficulty over the operation carried out in California is that it may have catered to the researchers' needs first and to the patient's only second. Over the past seven years, Dr Leonard Bailey, the surgeon heading the transplant team, has performed 150 such transplants on animals. There can be no doubt that the team was more than eager to try out its technique on a human. Dr Bailey's sincerity and his commitment to helping infants born with this and other tragic birth defects is above reproach. But it is fair to ask if this was the time to extend what Dr Bailey himself calls a "highly experimental" operation to humans.

It is not reassuring that the Loma Linda hospital did not even check with the California Regional Organ Procurement Agency to find out if a suitable human heart was available. (As it turned out, one was; it is not known whether it would have proved



compatible.) It is less reassuring that hospital spokesmen first said they had not known that a human heart was available, and then claimed that in any event it would have taken five days to match the tissues of donor and recipient. (According to researchers at Stanford University, a major centre for heart transplants, the procedure takes only eight hours.) Bailey then acknowledged that a human donor had not been sought because, simply, the purpose of his experiment was to see if the baboon heart would work.

Bailey has been given approval and has been promised support from Loma Linda to perform five more baboon-heart transplants on human subjects. One can only hope that before the next operation, the hospital will agree to release its protocols for the operation and copies of the informed consent forms that parents are asked to sign; so far the hospital has refused to do so. This is not nitpicking; although the research is privately supported, Loma Linda should feel an obligation to the scientific community if not to the public as a whole to provide reassurance. Otherwise suspicions will arise that its researchers are playing to the grandstands. □

French research

New minister plans for selectivity and flexibility

Paris

M. HUBERT Curien, newly-appointed minister of research and technology in France, is preparing to make French science policy really work.

For three years, French scientists have enjoyed close attention, encouragement and even cash from a government which saw — and still sees — science as the nation's salvation. But the economic results have been disappointing, and major government programmes in biotechnology and electronics, for example, have been criticized as substantial failures.

Publicly, Curien does not agree. ("It's difficult for a minister to admit failures", he said in his Paris office last week.) But within the ministry, he is making a careful assessment of the "*programmes mobilisateurs*" set in train by his predecessor, Jean-Pierre Chevènement, in 1982.

Selectivity is the watchword. The trouble with the early dreams was their breadth, all of biotechnology, for example, or all of electronics. But France has only 50 million people and, more tellingly, a history of success in narrow rather than broad technologies such as: nuclear power, space and the high-speed trains. Curien's task must now be to focus the programmes more narrowly.

Curien was previously president of the French space agency (CNES) and of the European Space Agency (ESA), and he recognizes the difficulty. "It is much easier to make good central policy in things like space, where there are few customers and, when you have half-a-dozen programmes, you've covered all the field."

Curien says that while the biotechnology programme did not achieve everything, it created a number of small companies and developed a view of what French industry needed to do. So Curien now has to choose. So far, he has picked agriculture and food, where many of the French traditional industries (such as wine and cheese) are both of great importance to the French economy and in very poor contact with French science. This must change "very rapidly" if the industry is to remain competitive.

In electronics, similarly, there must be rationalization and selection, he says. He cites as promising fields software, where France is already strong, robotics ("we've made some advances") and computer hardware where, says Curien, we should expect a new French product in the mid-range between a microcomputer and a minicomputer.

French researchers should also find themselves with more computers next year, according to Curien. He claims to have cut

the Gordian knot which effectively forbade French laboratories to buy foreign computers while the French industry struggled to catch up. This policy has led to serious under-equipment particularly in nuclear physics groups. But from next year, says Curien, groups will be free to buy computers which are "essential" for their laboratories if they will not be available "in the coming years" in France.

Curien is also seeking more money for industrial research, both from industry itself and from banks. "There is still a large gap between the [1982] plans and reality." France will have to make a "big effort" to catch up with Japan or even West Germany. In the big companies it is a question of rate; in the small, that they do no research at all. The big companies can be influenced from Paris but Curien says the government encouragement of regional science policy is beginning to have important effects on the smaller enterprises. Curien is also consulting the banks about legal obstacles to raising risk capital. In France, a person once bankrupt is forbidden from ever running a business again.

The minister also plans to raise the political temperature of science again next year (cooler now than in the Chevènement years) by staging a major debate on French

research and development in the National Assembly. The topic, ostensibly, will be that of what is to be done in 1985 when Chevènement's 1982 law on the orientation and support of French science comes to the end of its term. "It may be a new law, it may not", says Curien noncommittally. One possible theme is a linkage between the ministry of research and technology and the Administration du Plan, the French ministry that makes long-term "plans" for France with a considerable degree of independence from the president or prime minister — and a sometimes equal independence from effect.

Another key issue for Curien is the old chestnut of finding jobs for young researchers, or the ageing of the research community. So far France has not met the 4.5 per cent recruitment target of the 1982 law but has kept up a respectable 3 per cent; but even this cannot go on, says Curien. "We cannot solve this problem by creating new jobs every year. We must have a real policy." In 10 years the problem will change as the "recruitment bulge" retires but the bulge comes at different times in different agencies so that retirement and recruitment must be managed.

Curien says that making the scientists civil servants (in a new employment contract to be published soon) "will ease the situation". The point is that the new contract will give researchers "a parachute" — a guarantee of their old jobs back — if they shift into something new (such as industry). Curien hopes this will cause movement and, no doubt, that in the end not too many parachutes will have to be used.

Robert Walgate

West German research

Industry favoured by budget

Aachen

THE budget of the West German Ministry for Research and Technology, which forms 70 per cent of the total government civil research budget, is to be increased by 3 per cent in 1985. This is more than the growth in the national budget (1.2 per cent), and reflects the attempt of the government to keep abreast of new technological development.

The distribution of the budget of DM 7,250 million clearly demonstrates the government's intention to promote industrial research efforts. Whereas support for the reactor in Hamm has been reduced by 6.1 per cent, industry will be given increased support, both directly through a 28.5 per cent increase in research subsidies, and indirectly, through a reduction in taxes and financial support for research projects.

Particular encouragement is given to the employment of young scientists by companies with fewer than 3,000 employees and a yearly turnover of less than DM 300 million. Sixty per cent of the salaries of new research staff will be paid

for 15 months when these conditions are fulfilled. Exchange of younger scientific personnel from industrial research laboratories to academic laboratories will also be supported to stimulate transfer of information between the sectors.

In particular, the redistributed budget is designed to assist the foundation of more new technology-oriented companies for which an increase of financial support of nearly 150 per cent is foreseen. Directly supported research institutes, which absorb more than one-third of the whole budget, are expecting an increase of 5.3 per cent. Furthermore, Dr Heinz Riesenhuber, the Minister for Research and Technology, stresses the need for adequate research into the consequences of new technological developments. In the face of the effect of pollution on the forests in Germany, efforts are to be made to evaluate a wide range of developments, from the use of methanol as a fuel to the value of the electronics industry in improving the employment market. A 122 per cent increase in funds for such studies is anticipated.

Jürgen Neffe

NIH budget

Reagan vetoes busybody bill

Washington

EFFORTS by the US Congress to create a new arthritis institute and a new nursing institute at the National Institutes of Health (NIH) have been blocked. President Reagan last week vetoed a controversial NIH reauthorization bill passed in the last few days of the 98th Congress which, besides creating the new institutes, would have required the appointment of disease prevention directors and a formalized NIH policy on matters as different as the use of experimental animals and investigation of scientific fraud. Because Congress is not in session, the so-called "pocket veto" cannot be overridden. In his memorandum of disapproval, the president said the bill would create expensive new organizations, institutionalize over-specific research and place undue constraints on executive branch authorities.

The bill, which had received wide bipartisan support in Congress, was the culmination of a two-year effort by Representative Henry Waxman (Democrat, Los Angeles) to introduce more congressional control over NIH. Waxman last week described as "callous and insensitive" the president's veto of a bill that would "improve our ability to find a cure for cancer, arthritis and stroke".

The reaction at NIH, however, is likely to be one of unmitigated relief. While some of the bill's provisions would merely have added statutory authority to guidelines already in use, others were seen as unwarranted meddling in NIH's internal affairs. The creation of the new institutes was particularly opposed, on the grounds that it would introduce new administrative costs to little effect. The president's veto does not threaten existing NIH programmes, which can continue to operate (as they have since 1980) under the general authority of the Secretary of Health and Human Services to conduct and support research. Congressional aides said after last week's veto was announced that there is still widespread interest in reauthorizing NIH and that the veto will dissuade a future Congress from moving to pass similar legislation. Some predicted that parts of the now-dead bill will be resurrected in a new legislation next year.

The compromise version of the bill that eventually emerged from a House-Senate conference represented a considerable watering-down of Waxman's original proposals. The requirements placed upon institutes to undertake specified kinds of research would nevertheless have had an immediate impact in some instances. The bill would also have established a plethora of statutory bodies to oversee research in different areas. Present regulations on *in utero* fetal research would have been encoded in law, as would the requirement for peer review of research grants.

The bill had included a provision to set up a Congressional Ethics Advisory Commission to study human genetic engineering and fetal research, to have been modelled on Congress's Office of Technology Assessment. Despite last week's veto, preparations to establish an advisory committee of some sort are going ahead: it is not yet clear what sorts of legal authority are required for different functions of an advisory committee.

One of the few parts of the authorization bill that NIH is sorry to lose is that relating to use of experimental animals. The requirements were almost identical with new guidelines already being implemented by

NIH, but a legal obligation to follow the guidelines would have allowed NIH to defend itself more strongly against organizations protesting about the use of animals in research.

The separate appropriations bill for NIH also passed recently by Congress is still waiting for the president's signature. Congress awarded NIH \$5,100 million for fiscal year 1985, an increase of 14 per cent on the previous year, considerably more than the administration's request. There is however little reason for the president to veto the appropriations bill, because a back-up appropriation for NIH included in the continuing resolution provides an identical sum. For the time being, at least, it seems that NIH has succeeded in both having its cake and eating it.

Tim Beardsley

Molecular science

Japan's next target — biology

Tokyo

A THREE-YEAR project to develop in Japan a whole new set of automated tools for molecular biology and cancer diagnosis is about to be launched. The Science and Technology Agency (STA) hopes for rapid government approval for a plan to apply Japan's skills in electronics and precision manufacturing in this field.

Overall expenditure for the project is expected to run to around ¥2,000 million (\$8 million) with ¥618 million (\$2.6 million) allowed for in the first year. The money will come from the special co-ordination funds of the Council for Science and Technology, Japan's highest science policy-making body.

Announcement of the project comes just as Prime Minister Yasuhiro Nakasone has agreed to give top priority to cancer research in next year's budget. Since he gave his approval to a ten-year programme for cancer control last year, several of the science ministers have announced new plans — and new funds — for cancer research (see *Nature* 310, 264; 1984). Now, Nakasone seems to have given tacit approval to an overall 17.8 per cent increase in funds for the next fiscal year.

The overall chief of the new STA project will be Dr Toyozo Terashima, deputy director of the National Institute of Radiological Sciences. There are two main themes: technologies that can directly facilitate research into the molecular biology of cancer, and technologies of use in the diagnosis and therapy of cancer.

In the first theme, attempts will be made to speed up several routine but time-consuming procedures of the molecular biology laboratory. A good part of the research will be performed by industrial companies, in cooperation with university and government research institutes.

Watch-makers Seiko will continue development of an automatic DNA sequencing machine (*Nature* 307, 193;

1984), while film-makers Fuji Photo will improve their new gels for DNA electrophoresis. The chain of events may then be completed by electronics giant Hitachi, trying to develop computerized equipment to read the gel electrophoresis pattern.

Meanwhile, a large chemical manufacturer, Toya Soda, will develop equipment to extract specific DNA fragments from a DNA mixture and tackle the difficult task of building an automatic amino acid sequence analyser. And Japan Electron Optics Laboratory will attempt to develop technology to analyse the three-dimensional structure of protein-DNA complexes using electron beams.

Another goal is to find new ways of introducing DNA into cells: earlier this year, a group at STA's Institute of Physical and Chemical Research (RIKEN) developed a system for opening tiny holes in cell walls using a very brief laser pulse. In the one-second interval before the hole closes spontaneously, DNA from the surrounding medium can enter the cell. Further new systems, using both viruses and very short high-power electric pulses, are now to be tried out by a pharmaceutical company, Dai-ichi Seiyaku. RIKEN and the Tokyo Metropolitan Institute of Medical Sciences will also try new techniques to extract and purify very small amounts of protein produced with the proliferation of cancer cells. New assay cell culture systems for oncogenes will be developed at the Tokyo Medical and Dental University.

In the second part of the project, RIKEN and the National Institute of Radiological Sciences will commence work on the analysis and synthesis of glycoproteins located on the surface of cancer cells, and the Electrotechnical Laboratory on computer tomography for the detection of cancer. Finally, the huge Sumitomo Electric Company is to seek ways of speeding up production of monoclonal antibodies.

Alun Anderson

Genetic manipulation

NIH concede part of Rifkin suit

Washington

THE US government has quietly conceded a major claim in the lawsuit brought by anti-genetic-engineering activist Jeremy Rifkin to halt field trials of recombinant microorganisms.

Last May (see *Nature* 24 May, p.296), US District Judge John Sirica placed a preliminary injunction on all such experiments supported by the federal government, and in particular an experiment planned by Steven Lindow of the University of California at Berkeley to test the ability of genetically-engineered bacteria to protect potato plants against frost damage. Sirica ruled that in approving that experiment, the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH) had failed to satisfy two legal requirements: it had not conducted a formal environmental assessment of the experiment, and it had not prepared a much lengthier environmental impact statement on the entire programme of environmental release of recombinant DNA.

In an appeal filed with the US Circuit Court of Appeals in Washington, the government has now indicated that it will prepare an environmental assessment of the Lindow experiment and any others involving release to the environment. The government is continuing to appeal against Sirica's finding that what is called a "programmatic impact statement" is required. In a separate appeal, the University of Cali-

fornia is challenging both requirements.

According to Bernard Talbot, deputy director of the National Institute of Allergy and Infectious Diseases, the NIH institute that contains RAC, attorneys of the Department of Justice had pressed NIH to drop its opposition to the environmental assessment requirement, feeling that the government's case would be much stronger if it were limited to the issue of the future programme. The government argues that there is in fact no "programme" of release to the environment, and that the proposed experiments are so widely different that a case-by-case approach is the only one possible.

The Office of Recombinant DNA Activities at NIH has already begun to compile an environmental assessment of the Lindow experiment, but NIH officials make it clear that they consider the exercise meaningless, fulfilling a formal legal requirement without adding anything of substance. The Lindow experiment was debated at two public meetings of RAC, and was revised in response to concern expressed by the committee. NIH appear to be hedging their bets by agreeing to go ahead with the environmental assessments at this stage. And with the University of California continuing to press the full appeal, the government clearly had little to lose.

Although Sirica's injunction specifically exempted experimental proposals from commercial companies — which have been

What, no uranium?

Canberra

AUSTRALIAN exports of uranium oxide to France are likely to stop for at least two years. The Australian Minister for Resources and Energy, Senator Walsh, announced last month that the first eight shipments of yellowcake that were to have been sent to the French power company Electricité de France under a contract with the Nabarlek uranium producer, Queensland Mines Limited, will be deferred because of the continuation of nuclear testing at the French underground site at Mururoa Atoll, in the south central Pacific.

This is in line with the Australian government's ban on uranium sales to France confirmed during the Labor Party's national conference in Canberra in July, when a vote to continue mining and export of uranium was carried (see *Nature* 2 August, p.354). The first shipment (due in October) would have been 100 tonnes of yellowcake valued at \$A6 million. The Australian government has agreed to purchase the first eight contracted shipments at the original price, but Queensland Mines is obliged to repurchase the uranium "if circumstances permit". This is interpreted to mean if and when the French stop testing nuclear weapons in the Pacific.

Jeffrey Sellar

submitting them to RAC for approval on a voluntary basis only — NIH director James Wyngaarden has ordered that environmental assessments be carried out for these as well. At its June meeting, RAC approved two commercial proposals from Advanced Genetic Sciences (AGS) — almost identical to the Lindow experiment. Once the Lindow document is completed, it should be a quick matter to prepare for the AGS proposal, and that experiment should be clear to go ahead by next spring.

The appeals court is scheduled to hear the case on 5 December. Although the appeal is technically on the preliminary injunction only, for practical purposes the court's decision will decide the entire case. If the appeals court upholds Sirica's decision, the government will probably concede the case rather than let it drag on to a near-certain outcome.

Rifkin was notably unsuccessful on another front last week, RAC unanimously rejected his proposal to ban all transfers of genetic material from one mammalian species to the germline of another. The committee noted the benefits that such research holds for the study of cancer and other diseases. Researchers at the University of Pennsylvania have already successfully inserted the gene coding for human growth hormone into mice embryos, and are planning similar experiments on sheep; RAC also noted the possible benefits that such research would bring in more efficient food production.

Stephen Budiansky

Gloomy outlook for young neuroscientists

Los Angeles

As the US Society for Neuroscience held its 14th annual meeting in Anaheim from 10 to 15 October, there was talk in the corridors of great frustrations and dashed expectations.

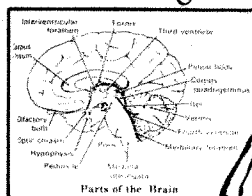
But the subject did not revolve around difficult research questions in neuroscience. Rather, it applied to scores of young scientists who are dropping out of the discipline because financial support and job prospects are woefully inadequate.

Neuroscientists are not the only group to complain about support for the students they train. But, argued Dr Dominick Purpura, last year's society president, because neuroscience is reaping great intellectual rewards, it is attracting many of the brightest and best students in US universities.

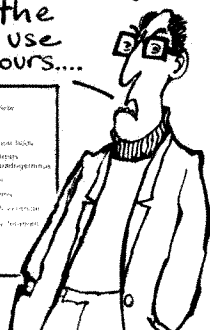
Once trained, the young neuroscientist is faced with "incredible competition", Dr Purpura said. "Many universities will take on a young scientist for one or two years, whereupon the scientist must provide his or her own financial support. But the young are competing against established people for limited funds. There is so much compe-

tion that people are disheartened and drop out." The job placement centre at the meeting interviewed 1,200 candidates for fewer than 250 jobs, mostly postdoctoral appointments without long-term security.

Unfortunately, few of you will be given the opportunity to use yours....



BIRCH



The National Institutes of Health and the National Science Foundation together support 85 per cent of research in biology and biomedicine, Dr Purpura said. "They are \$50 to \$100 million short in grants that might allow young neuroscientists to get a start."

Sandra Blakeslee

Japanese technology

MITI seeks friends from abroad

Tokyo

JAPAN'S Ministry of International Trade and Industry (MITI) last week took the remarkable step of inviting foreign representatives to attend a joint meeting of two of its key councils concerned with the planning of research and development projects.

A major new bill to promote high technology research is in preparation (see *Nature* 310, 614; 1984), and MITI seems anxious to sound foreign opinion before presenting the bill to the *Diet*. What remains unclear is whether the meeting will turn out to have been a sop to those feeling threatened by MITI's new plans or a first step in an initiative to encourage international research cooperation. For the foreigners, at least, the hope was that this would be the first of many meetings.

The two councils, the Industrial Technology Council and the Industrial Structure Council, between them play the major role in deciding the thrust of MITI's big research and development projects. All too often, these projects have been fiercely criticized by foreign governments for "targeting", or for aiming to use the combined strength of government and industry to achieve Japanese technological domination in some highly profitable area.

Recent projects have evoked strong reactions worldwide. Those under way at the moment include the fifth generation computer project (which has frightened many advanced nations into launching their own "artificial intelligence" programmes); an optoelectronics project designed to rid optical fibre circuits of relay electronics through the use of optical switching; the massive sunshine and moonlight projects intended to exploit new energy sources (geothermal, solar and biomass) and to find new ways of conserving and storing energy; a host of projects on improving industrial performance through use of robots, flexible manufacturing systems equipped with lasers and computerized sewing and garment assembly machines; and long-term projects aimed at new ceramics and superlattice semiconductors.

The first MITI project that really evoked foreign discontent, however, was the VLSI (very large scale integrated circuit) project that ran from 1976 to 1980 and which involved 100 researchers from the seven large computer manufacturers, MITI's own electrotechnical laboratory and the telecommunications monopoly NTT's electrocommunications laboratory. Virtually all aspects of the production of microchips were investigated, seven hundred new inventions were patented and within a year of the end of the project, Japanese manufacturers had seized 70 per cent of the world market in 64K random access memories (rams) and were leading world

research in the production of 256K rams and 1 megabit microchips.

US electronics companies sent reeling by this assault on their markets vigorously protested that the pooling of competing companies' research resources was unfair — particularly as their own anti-trust laws would have made a similar strategy impossible. Japanese companies on the other hand show little respect for the value of the MITI project, and instead attribute success in the world microchip market not to a mythical "Japan Inc." but to the fierce competition among themselves to produce the finished item as cheaply as possible.

Since that time, there have been regular demands from the United States to allow foreigners to act as witnesses, or even participants, on Japanese government research councils. While this may seem unreasonable interference in another government's deliberations, the United States says it seeks only a clear understanding of new legislation before it can cause industrial hostilities. In Japan that means seeing the legislation before it is submitted to the *Diet* which can hardly be considered a major forum for debate when more than

eighty per cent of bills pass without any amendment whatsoever. (The general procedure is that a consensus is worked out behind the scenes before a bill is ever presented.)

In the event, the representatives invited to the MITI councils' meetings — from the United States, United Kingdom, West Germany and France — were from their respective Chambers of Commerce rather than from embassies. MITI itself made no specific proposals, although suggestions have been made that foreign companies operating in Japan may be allowed to use the new industrial technology centre that MITI wants to set up, and that exchanges of personnel may be encouraged in areas in which Japan now leads the world. Foreign representatives were however, given a chance to say how international cooperation might be brought about.

Other attempts by Japan to encourage major international research projects have so far yielded little fruit. The series of proposals made at the Williamsburg summit, such as that for the development of new robots to work in dangerous environments, may have hung fire because they tried to involve all the summit nations. Smaller scale projects arrived at through bilateral agreement may prove more successful.

Alun Anderson

Britain in space

Strong links with Europe urged

BRITISH scientists and grant-making agencies are urged to play a fuller part in the work of the European Space Agency (ESA) in the report of a committee to consider future policy. The committee, under Sir Mark Richmond, the vice-chancellor of the University of Manchester, also says that its parent, the Science and Engineering Research Council, should take the initiative in persuading government departments to set up a strong coordinating body for British space research.

Among academics working in the field, the overwhelming reaction is relief that the Richmond committee has not advocated a retreat from space research. The same interests complain that the committee has not openly advocated some way of financing space research other than by subventions from the academic research budget. No doubt the committee would have been more explicit if it had some inkling of what the British government would be prepared to spend (if anything).

The origin of the committee, reflected in the arguments of the report now published, is the financial pressure of British space research in recent years. The committee points out that British government spending in the civil field is £80 million a year, compared with £200 million a year in West Germany and £300 million a year in France (the main sponsor of the Ariane launcher).

The committee notes that British scientists have in the past been able to sustain

their research by means of bilateral agreements, chiefly with the United States, largely on the strength of good science and competence in the design and construction of instruments. But this period, the committee says, is drawing to an end, partly because British scientists are no longer outstanding among Europeans, partly because they have less to offer in cash as well as kind, partly because of some recent failures of instruments built for other people's satellites (such as some of the detectors of the EXOSAT satellite) and partly because of a change of US policy, now slanted towards collaboration with ESA wherever possible.

The Richmond committee acknowledges the relatively high cost of international collaboration but says that if British academic science does not intend to abandon space altogether, it should participate more fully in ESA. On particular points, the committee recommends a transfer of the Science and Engineering Research Council's interests in remote sensing by satellite to the Natural Environment Research Council and the encouragement of British industry to interest itself in microgravity experiments.

The Richmond report has not been formally adopted by the Science and Engineering Research Council. The council is probably as embarrassed as the committee by uncertainty about the government's financial intentions. □

European space

Another Franco-German deal

Paris

FRANCE and West Germany have reached "a very good agreement" on collaboration in space and on the European response to the invitation to participate in the US space station, French science minister Hubert Curien said last week.

No figures were discussed between Curien and his German opposite number, Heinz Reisenhuber, when they met in Bonn on Wednesday, but there was agreement in principle that the French should join in with the "Columbus" space station project proposed by Germany and Italy, while Germany would take a share in the HM60 cryogenic motor under development in France for the Ariane-5 space launcher.

Thus it seems that a continental, Franco-German-Italian plan has emerged for a space programme for the European industry in the 1990s, with the other potential major partner, Britain, left on the sidelines. Curien was "sure", however, that Britain would participate "though I'm not certain at what percentage". British government sources have said that the nominal 2 per cent that Britain took in Ariane would certainly be too small.

But whatever share Britain takes, it is clear that European mainland is now calling the tune. No British proposals have emerged that could be traded against Columbus and Ariane-5 to provide a package more suited to Britain. British Aerospace's HOTOL concept of an air-breathing, runway-using hybrid between the space shuttle and Concorde is too distant a prospect to be of use in negotiations, and the proposed free-flying robotic platform may be too like parts of Columbus.

Meanwhile, Europe's space scientists are attempting a defence of their territory, the mandatory science budget of the European Space Agency. In principle, the space station is part of ESA's optional programme. Microgravity and Earth resources research are also optional, coming under the transportation and applications budgets, but the "traditional" space sciences of high-energy astronomy, magnetospheric research and planetary sciences are mandatory to ESA members. Space scientists in this traditional area fear an erosion of mandatory budget — whereas in fact it has long been claimed that it should be increased.

Just by chance, the ESA space science directorate has now come forward with a rational plan as to how that budget should grow. Called the "Horizon 2000" study, it was prepared from 77 responses from the larger space science institutes around Europe to a request for realistic projects for the 1990s. Whittled down by an ESA committee, it is a proposal for a 50 per cent growth in real terms over eight years. The

programme would be based on four cornerstones:

- A solar terrestrial programme (space plasmas)
- A mission to asteroids and comets including return of material to Earth
- A high throughput X-ray mission for spectroscopic studies at 0.1–20 keV
- A high-throughput heterodyne spectroscopy mission to cover the last remaining unexplored region of the electromagnetic spectrum.

Mrs Indira Gandhi

A sharp-witted democrat

Mrs Indira Gandhi, killed last week, had the sharpest wit. I met her at the end of February when her attempt to negotiate a settlement of the Sikh rebellion with community leaders from the Punjab had collapsed. It would have been forgivable that she should have cancelled a meeting with a visiting journalist interested only in her expectations of Indian science. Perhaps she welcomed a chance to talk about something completely different.

Disconcertingly, this did not prevent her from reading and signing her mail while keeping command of the conversation. Occasionally, members of her staff would creep into her office with messages and



would be told what to do by signals, a movement of the hand or eye that did not interfere with the flow of words.

There was an awkward moment at the beginning, when it seemed as if the Prime Minister thought that *Nature* is a magazine devoted to environmental causes; she launched on an eloquent speech about the beauty of the Indian landscape that threatened to use up the 45 minutes available. But, when prompted, she changed the course of her conversation without batting an eyelid.

Quite apart from Mrs Gandhi's belief (taken over from her father) in the importance of research and development in India, she exuded affection for the field and its practitioners — "my scientists" she called them. Her gratitude to people like Professor M.G.K. Menon (who has held

Such missions would not be in competition with United States or Japanese projects, it is claimed, but could be part of a balanced world programme of space research.

The Horizon 2000 study has been very well received in West Germany and Switzerland, where both countries are willing to provide the 7 per cent budget increase a year it implies, but other ESA members are proving more reluctant. M. Curien, for France, says he might find 5 per cent "at a great stretch", and a clutch of other states are talking of a mere 3 per cent. Britain has nothing to say just yet.

Robert Walgate

almost every senior scientific post in the government that there is) was evident. So was her pleasure at having as colleagues people such as Dr T. Koshoo, now Secretary at the Department of the Environment, whom she had inveigled to Delhi over a period of two years.

Mrs Gandhi was astoundingly knowledgeable about the vast programme of research her government mounted. Her willingness to visit laboratories and construction sites was renowned. One consequence was the high morale of those who worked there.

With some of her problems, Mrs Gandhi was plainly impatient. University administrations had let authority slip from their grasp, she implied; they should be more vigorous, more like her perhaps. But then, visitors from overseas should beware of the trap of applying in India the standards conventional in the West. The real wisdom of India rests with the village peasants, hardly any of whom can hope that their children will find their way to a university. It was easy to see how other Indian politicians, allies and critics alike, acknowledged that her electoral strength lay in the villages.

So how to reconcile the poverty with India's ambitions in high technology — nuclear power, space technology, electronics and so on? Mrs Gandhi was eloquent on the theme that a sovereign state as powerful as India could not allow itself to remain in thrall to the West. "Look at what they did to us over the fuel for Tarapur", a reference to the ham-handed administration of the US Anti-Proliferation Act towards the end of the Carter presidency.

Mrs Gandhi's demeanour throughout this conversation was in my experience extraordinary. She was direct to the point of being unguarded; no doubt she had heard it all before, but she gave no hint of calculating what to say. She was practised at tormenting the British about colonial rule, yet able to talk as a woman of the world. She gave no sign of trading on her political power. Manners aside, her courtesy was her frankness.

John Maddox

Agricultural research

International centres under stress

Washington

THE Consultative Group on International Agricultural Research (CGIAR) meets in Washington this week to decide how the 13 research centres under its control are to respond to continued financial uncertainty. Exchange rate losses caused by the increased value of the dollar, and the growing tendency of donors to support specific programmes judged to bring local benefits rather than backing core research, have meant that the heady expansion of the 1970s has been all but forgotten.

The centres had a total nominal budget last year of \$180 million, but the failure of some donors to meet in full their commitments to the centres has, for several years running, left programmes high and dry. For 1985, the CGIAR technical advisory committee is requesting a total budget of between \$187 million and \$199 million, an increase of 7–14 per cent on the current year, but actual reductions in 1985 are not impossible. The traditional supporters of CGIAR — of which the United States is the largest — are under internal pressure to restrict multilateral aid in favour of support for bilateral projects, whereas the newer donors have not yet been able to make a substantial impact. The World Bank remains the donor of last resort, paying the bills that others have failed to meet.

The Rockefeller Foundation, which founded the first of the agricultural research centres (for breeding wheat, in Mexico) and the Ford Foundation, a substantial sponsor from its early days, have been reducing their contributions to the centres in recent years. Rockefeller is turning instead to centres outside the CGIAR system and is to announce a major initiative in plant cellular and molecular biology before the end of the year, probably based in Mexico. The budget will be in the region of \$5 million a year, but already some are asking whether the Rockefeller Foundation will be able to recruit to Mexico enough high-calibre scientists to justify this outlay.

Despite the financial uncertainty, CGIAR appears confident that most of the centres are still doing a good job. But there is frustration over the plight of some African centres, which have to work with often under-developed national programmes. Local interests seek to direct research efforts towards strictly local problems, an approach which CGIAR feels to be unproductive in the long term. The centres which have historically been the most effective seem to be those with a strictly defined mandate, whereas attempts at some centres to work on a number of different local problems are held to have been less productive.

The International Centre for Agricultural Research in Dry Areas, in Syria, and

the International Livestock Centre for Africa, in Ethiopia, are likely to come under particular scrutiny this week, and there may be attempts to tighten up their programmes. Particular attention will also be paid to the West Africa Rice Development Association (WARDA) in Liberia, which has been under heavy criticism. The future of WARDA within the CGIAR system appears still to be open to doubt, despite some attempts at reform by the WARDA management.

CGIAR has at various times been criticized for advancing the careers of scientists

rather than the fortunes of the developing countries it is intended to benefit. The criticism is apparently taken seriously, to judge from the number of introspective studies by CGIAR now in progress. Should its centres, for example, try to stand in place of national agricultural development programmes where these are seen to be inadequate? There was some agreement in preliminary discussions last week that centres in different parts of the world should be ready to adapt their strategies to local circumstances despite the dangers. There was also an agreement to adopt a plan to foster cooperation between the various institutes, including a joint training programme in farming systems research.

Tim Beardsley

Early success a problem for CGIAR

Washington

THE international research centres supported by CGIAR stem from a crop improvement programme undertaken in the 1940s by the Mexican government and the Rockefeller Foundation. So successful was the project that in 1959 Rockefeller joined forces with the Ford Foundation to establish the first international agricultural research centre, the International Rice Research Centre in the Philippines. In 1966 the two foundations sponsored the second centre, Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT) in Mexico, with two more centres following the next year.

By the late 1960s Rockefeller and Ford were each contributing \$3 million annually to the centres, and it became clear they could support no further expansion. In 1971 CGIAR was created at the suggestion of the World Bank to coordinate finances of the centres, which now number 13 in all.

CGIAR, which has a full-time secretariat provided by the World Bank in Washington, remains a completely informal organization with no constitution or charter: its decisions are reached only by consensus among its members.

Funding has changed drastically over the past decade. The US Agency for International Development is now the largest contributor (\$44.5 million in 1983), followed by the World Bank (\$19 million). The International Development Bank contributed \$8 million. The Rockefeller and Ford Foundations have both decreased their contributions recently together accounting for less than \$2 million last year.

But the difficulty for CGIAR now seems to be in living up to the early successes of the first institutes: by the late 1970s Mexican wheat was grown on 29 million hectares of land worldwide, and improved rice strains on 25 million hectares.

Tim Beardsley

Nature index of biotechnology stocks

12-Month high	12-Month low	Company	Close previous month	Close 26 October	Change
14	6	Biogen (Switzerland)	8	7	- 1
2	1	Bio-Logicals (Canada)	1 1/4	1 5/16	+ 1/16
14 3/8	5 1/8	Bio-Response (USA)	7 3/4	6	-1 3/4
14	9 1/4	Cetus (USA)	11	10 1/4	- 3/4
10 3/8	4 1/4	Collaborative Research (USA)	5 5/8	4 1/2	-1 1/8
19 7/8	11 1/2	Damon (USA)	14 3/8	12 1/8	-2 1/2
26 1/4	11 3/4	Enzo-Biochem (USA)	14 1/2	18 1/4	+ 3 3/4
10 1/8	4	Flow General (USA)	5 1/4	4 7/8	- 3/8
42 1/4	28 3/4	Genentech (USA)	30 1/4	9 7/8	-1 3/8
10 3/4	4 1/2	Genetic Systems (USA)	6 3/4	6 1/2	- 1/4
17 1/4	6 3/4	Genex (USA)	9 3/4	7 1/8	-2 5/8
23	11	Hybritech (USA)	13 1/4	15 3/4	+ 2 1/2
16 1/4	6 1/4	Molecular Genetics (USA)	7 3/4	7	- 3/4
15 1/2	8 3/4	Monoclonal Antibodies (USA)	10 3/4	10 1/2	- 1/4
60 7/8	20 7/8	Novo Industri A/S (Denmark)	33 3/8	20 7/8	-12 1/2
22 3/4	14 1/2	Pharmacia (Sweden)	17 7/8	16 1/4	-1 5/8

Closing prices are for the last Friday of the month. For over-the-counter stocks, bid price is quoted; for stocks on the American and New York exchanges, the transaction price. *Nature's* weighted index of biotechnology stocks stood at 121 on 26 October, compared with 139 a month earlier. Data from E.F. Hutton, Inc.

Confusion about Chinese names

SIR — Richard J. Wang's note "Xiaoping first?" (308, 768; 1984) only scratched the surface of the issue of Chinese family names in romanization. In the five articles detailing results of the French-Chinese study of the North Himalaya-Tibet area (308, 17-36; 1984), only the second has authors' names correctly abbreviated in the table of contents. Admittedly, it is difficult for the uninitiated to know (or even make an educated guess) at which is the family name.

Confusion begins because the Chinese custom in Chinese is to put family name first, the opposite of the European custom. Added confusion comes because in romanization some Chinese westernize name order (family name last) while others do not. Further, some Chinese hyphenate the *given* name because it is two-character (confusing at first because Europeans sometimes take a hyphenated *family* name), while others (particularly in mainland China) fuse the romanization into one "word". Still others make three separate words for their name, so that the given name would be two words, always to be used together. This last practice is the most confusing since there is no cue to guess the family name; in the other methods, once realized, the hyphen, or the length of the word, allows the given, and thus the family, name to be identified. Even if one is familiar with the common Chinese family names, there are several distinct, widely-used, romanization systems (so Xu = Hsu = Shyu). Further, while pinyin is the sole romanization taught in mainland China, many Chinese elsewhere are not taught any particular system at all, so they sometimes show noticeable creativity. Knowing common last names in one system certainly helps, but not all the time.

The issue raised by all this confusion, particularly in the professional literature, is much more than that of slighting persons or cultures. It can and has already created a mess in indices. For example, to look up articles by Li Tindong in an index or abstracting service, does one look up L. Tindong, as given in that *Nature* table of contents and index. Or is it T. Li? I do not think it would happen, but if an editor insisted on consistency, it could also become T.D. Li, or T.-d. Li, or T.-D. Li. What is one to do? Even the thought of tediously searching each alternative is tiring, not to mention expensive if done through computer hook-up.

As an international journal, *Nature* should not be making these mistakes. Even after Mr Wang's letter I found an article by Zhang Yun (309, 547; 1984) listed in the table of contents and the mid-year index (310, 26 July 84) as Yun, Z. Poor Professor Zhang! Filed under first name. Ignorance could be claimed legitimately by my next-door neighbour, but by *Nature*? Clear, fair instructions to the authors, and clear, fair

editorial rules need to be established to handle these problems. Tell authors to circle family names, or, to indicate that last name, with or without hyphen, will be assumed familial unless otherwise indicated. Then give an example. Errors could be picked up, such as where R. Xu (in the first of the five articles mentioned) inverted his name apparently only because it is last in the list, even though it appears *Nature's* policy not to invert name order because of placement in a list of authorship. Consultation with librarians at an institute for Asian studies might help. After all, *Nature* does not abbreviate L.M. Van Valen as L.M.V. Valen (table of contents 5 January 1984).

I also suggest that indices should include the full romanized Chinese name. There are strong if not compelling reasons to support it. First is the lack of diversity in Chinese family names. Anyone familiar with Chinese names knows how widespread are a small number of family names such as Wang, Chen, Li, Zhou and so on. Indeed, in romanization the situation is worse than in Chinese because homophones (same sound and tone, different character) are so common. In addition, widely-used romanizations do not distinguish total differences, causing further redundancy. The only mitigating factor is

that use of different romanization schemes adds some variability. Chinese given names are quite varied, but all the points on romanization apply here as well. The habit of complete fusing of the given name further reduces variability when the given name is initialized. So the combination of initialling and the commonness of most family names must lead to immense redundancy.

Finally, Chinese names are short. Most are three characters — one familial, two for the given name — a few such as Zhang Yun are two, though a few others are four — for each name. Thus, one would be hard-pressed to find a romanized Chinese name over 15 letters. This is not true for names in many other languages, for example N.C. Vamvakopoulos (table of contents, 5 January 1984). *Nature* has recognized this since Chinese names are given in full at the top of all articles. The policy needs to be extended, perhaps not to the table of contents (so people know the family name!), but definitely to indexes.

With the increasingly large numbers of Chinese names coming into the western scientific literature, there are wide ramifications for information retrieval, not to mention human dignity.

JAMIE HOOK

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Student fees in Britain

SIR — When a nation is under financial strain, economies are necessary even in an important sector such as scientific research, which in the long run is one of the best investments. One could go further, admitting that financial restrictions, to some extent, may even be beneficial to research, in that they stimulate competition, force a better selection of people and projects and favour a more rational utilization of equipment and resources.

There is one measure, however, to which I would like to call your readers' attention, namely, the higher fees imposed on university students who are not resident in the United Kingdom. Apart from any question of reciprocity (I do not think that British students are requested to pay higher fees in any foreign university), I think that such a rule will ultimately damage British science. I know a case, by no means unique, of a British subject, not resident in the United Kingdom, who, having graduated *cum laude* abroad, wanted to study for a PhD in Britain. She would have been accepted by two leading university departments but at a cost of several thousand pounds. As a result, she accepted a research assistantship leading to a PhD, with an adequate salary, in a department of excellent reputation in another country in the European Community.

This is only one example of how foreign

students and graduates are actually discouraged from coming to Britain. Gifted students at least, especially at postgraduate level, should be attracted, even at the cost of some money. First of all, graduate students do productive work, which would be very cheap indeed if the nation has not borne the cost of their previous support and education. Secondly, when returning home these people will take away with them for life the memory of the country and of the institutions in which they were trained. This will result in the arrival of other young people and in the selling of equipment and may have even greater political implications. Last but not least, many of the best will stay. In this context, there is no need to mention what the United States has gained by recruiting foreign scholars and scientists, often in their youth.

I hope this will not be considered as an undue intrusion into the affairs of a country where I often was and am currently a guest. Indeed, for the gratitude I have for what I learnt and received in your country I felt a moral obligation to express a disinterested opinion on what seems to me a very serious matter.

FIORENZO STIRPE

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Civil aviation

SIR — Your leading article on 6 September, p.1 ("Poor show for aviation industry") complains that "civil air transport is not as accessible or cheap as it should be". Perhaps the judgement would be fairer had the article ended "as I would like it to be". The author has clearly not grasped the complexity of an airline operation. The technology necessary to take people around the world near or above the speed of sound costs money, and to do so safely requires one of the most intricate man-made systems most of us encounter. Add to this the requirement to ensure that flying is a positive experience for the customer and that there is a reasonable profit and to cope with political pressures from many governments.

As a pilot with a major international carrier I would hesitate to comment publicly on the financial wisdom of subcontracting aircraft component manufacture, an area which I recognize as only indirectly related to my area of competence.

R.J. ANDERSSON

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Indian science

SIR — I would like to add to the explanations which have been given in your columns of the poor quality of research in Indian universities and institutes. The problem at many Indian universities and institutions is a lack of understanding concerning the philosophy of higher education and a lack of sincerity on the part of the academic faculty and administrative staff. There is also a perversion of the professor-student relationship into what often resembles a master-servant relationship.

Many senior scientists from Indian universities and institutes work hard while in the West but when they return to India depend heavily on technicians and students in their laboratories. Another contributing factor is that scientists plan frequent long trips to the Western institutions. Their time in India is mostly spent on collecting money and making arrangements for their next trip. This situation deprives doctoral students of proper guidance from their professors. Often dissertations are presented to the university by the doctoral students in the absence of their major professor.

The concept of higher education in many Indian universities is misunderstood. Comprehensive solutions are required. Despite a huge cut in government support for scientific research in Europe and the United States during the past few years, the number of publications in a scientific journals is increasing. On the other hand, the Indian Government is spending a much greater percentage of the gross national product on science and technology than in

many other nations. Scientists and administrators in India cannot simply sit in their offices cursing the poverty of the Indian economy. In my opinion, the solution lies in increasing the commitment of all those involved in science.

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Scientists' oath?

SIR — Television dramas such as *Threads* and *The Day After* make us painfully aware of the great power that scientific and technical knowledge has for evil as well as good. A crucial question is the extent to which scientists, as individuals and as a profession, accept social and ethical responsibility for their research. I suggest that scientists, on graduation, take an oath or make an understanding that their work be for the benefit, rather than detriment, of mankind. Though such an oath would not legally prevent scientists from working on, for example, new nerve gases and new nuclear warhead systems, it might make a start to the establishment of a code of ethics for the scientific profession and bring this body into line with older institutions such as medicine and the church. Such a scientific Hippocratic oath might influence decisions made by individual scientists and a scientific code of ethics might answer an increasingly more popular groundswell of opinion that scientific "progress" *in toto* is not worth the associated risks to the survival of the human species.

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Jobs for biochemists

SIR — Richard Pearson's article "Is all science vocational?" (*Nature* 310, 260; 1984) will no doubt be widely read and quoted. As regards the employment of biochemistry graduates, however, the figures obtained by the Biochemical Society, which has been conducting an annual employment survey for some years, indicate a somewhat less gloomy situation. Thus, in 1983, 12.4 per cent of new graduates were still unemployed at the end of the year with another 6.3 per cent unknown as regards employment. At least 80 per cent are known to have found useful occupation within 6 months of graduating, such as further biochemical training for higher degrees (25.4 per cent), biochemical employment (25.5 per cent), other further studies (9.7 per cent) or non-biochemical employment (9 per cent). In addition, 11.7 per cent went abroad, including overseas students returning home and British students taking jobs. Given the present general difficulties as regards employment

of both graduates and non-graduates, these figures suggest that prospects for biochemistry graduates remain reasonably good and apparently considerably better than for biologists.

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RICHARD PEARSON REPLIES:

Professor Arnstein's letter provides valuable information about biochemists and highlights the range of employment they have entered in the past. However when making comparisons *between* subjects for example with biologists, it is important to compare like with like and to use common databases as was done in my article. While the statistics are capable of different interpretations, cross-referencing one source to another can lead to inappropriate comparisons.

Animal antibiotics

SIR — In the article by Stephen Budiansky on the emotive subject both sides of the Atlantic, "Are antibiotics in animal feed a threat to human health?" (*Nature* 4 October, p.407), a few statements cannot pass unchallenged.

For instance, the statement* that "none of the bacteria residing in the gut of farm animals is resistant" is just not true¹. Rather than looking for evidence that animal pathogens constitute the source of R plasmids for human pathogens, as is suggested, evidence already points to the R plasmid-carrying indigenous flora in the animal gut as the main reservoir². This reservoir is being selected continuously by the use of antibiotics for whatever purpose. The present debate, therefore, hinges on whether or not subinhibitory, rather than prophylactic or therapeutic, levels of antibiotics play the major role.

Further, the suggestion that the energy cost to bacteria of carrying R plasmids places them at a disadvantage is not scientifically substantiated. It is now recognized that many plasmids are stable entities of the bacterial cell. Also, antibiotic resistance determinants are sometimes genetically linked on the same plasmid with virulence determinants³ and consequently antibiotics select for both types of determinants.

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*This should have read "not all of the bacteria..." but was inadvertently changed in the editing. — Editor, *Nature*.

AIDS casts a longer shadow

The good news is of rapid progress in describing what seems to be a new virus — the bad news, proof of heterosexual transmission and of the mounting prevalence of antibodies.

WHILE no patient has yet been cured of the disease called AIDS (for acquired immune deficiency syndrome), it is proper that there should be full-hearted celebration at what has been done to understand the mechanism of this infection. That is one legitimate response to the appearance of the latest contribution from Dr Robert Gallo, at the National Cancer Institute, and his associates there and elsewhere, in France as well as in the United States (see page 166, this issue). The latest development is that Gallo has been able to clone DNA copies of the RNA genome of the virus, called HTLV-III (for human T-cell leukaemia virus), which was identified as the causative agent of AIDS only a few months ago. Presumably it is now a matter of weeks, not months, before the full nucleotide sequence of the 10,000 or so bases in the genome is known. Quite soon, it should also be known whether the vaccines against AIDS will be effective.

So quick has been the succession of events in this saga that it is easy to forget that the disease was first recognized only as recently as 1981. Inevitably, hindsight showed that the disease was not entirely novel — people had been dying from it for at least a couple of years. The chilling feature of AIDS was, and remains, the high mortality among those affected. In advanced societies, there is still no basis on which to deny that those frankly infected will in due course die, usually of some other infection or from Kaposi's sarcoma. That the disease, for the time being, is largely confined to a few groups of people (male homosexuals, those who inject themselves with narcotics and those dependent on blood transfusions, haemophiliacs especially) does not make the social problem less serious or even allow those not in one of the groups at risk to congratulate themselves that they will never come to harm. If, as is probable, HTLV-III is a virus to which the populations of advanced societies are still naive, it is understandable that it should first infect those to whom transmission is easiest. By the same logic, spread will eventually be more general. AIDS should be everybody's worry.

That is another reason for marvelling at the speed with which so much has been learned about the virus, and at the luck that AIDS did not strike ten years earlier, when it would have been much more difficult to disentangle what is going on. One of the crucial steps in characterizing the disease has been the recognition that an almost

invariable accompaniment of overt AIDS is an altered balance between the T lymphocytes called "helpers" and "suppressors". (There are fewer helpers.) These white blood cells are concerned with the regulation of the immune response in ways that are by no means clear. But ten years ago, even the distinction between helper and suppressor cells had not been established.

For the rest, the development of the present understanding of AIDS, and the description of HTLV-III, has followed the path that would have been expected of classical virology — but at an enormously accelerated pace. Gallo's group was able, in 1981, to hit the ground running because of the experience it had acquired in its investigation of the virus now known as HTLV-I, responsible for the disease called adult T-cell leukaemia, first recognized in south-west Japan but then among Caribbean and some African populations. That also upsets the balance between helper and suppressor cells, so that it is not surprising that Gallo and his associates can now also report that the genetic structures of the two viruses have much in common but are also distinct. If that investigation had not been fresh in their minds, it is hard to think that Gallo's group could have made such rapid progress.

But Gallo has not been the only investigator in the field. Professor Louis Montagnier's group at the Institute Pasteur in Paris was formally the first to announce that AIDS is caused by a distinctive virus, in May 1983. That virus, christened LAV, has now been shown to be indistinguishable from HTLV-III, and is also being produced by an infected cultured cell-line. There is even said to be some tension between the two groups. In August this year, Professor Montagnier wrote (see *Nature* 310, 446) in terms suggesting that he and his associates feared they would be overlooked in all the attention being paid to Gallo's fast-moving high-tech enterprise. That would be absurd as well as unjust, for in spite of what has been learned, so much remains to be learned about the biology of AIDS that everybody's energies will be needed before we are safe.

Mercifully, there is movement even towards that goal. Gallo and his associates have reported (*Science* 226, 447; 1984) the presence of HTLV-III in the saliva of apparently healthy male homosexuals and in that of people suffering from what is

called ARC, or AIDS-related complex (which may or may not be an early form of the disease). They have also recovered HTLV-III from the semen of two AIDS patients (*ibid.*, p.449). Guesses at the method of transmission by male homosexuals are thus confirmed; the bad news is that apparently healthy people can be carriers (and presumably transmitters).

It remains perplexing that so little can at present be made of the prevalence of antibodies against HTLV-III among apparently healthy people. Most AIDS patients carry antibodies against the virus, suggesting that their first response to infection was normal, but that their immune system was eventually overwhelmed. There seems to be abundant evidence (little of it published as yet) that the presence of antibodies is widespread among the groups at risk. Only a proportion of those infected by HTLV-III may succumb to AIDS, but, with the long lapse of time (perhaps four years) between infection and overt disease, it is not yet possible to tell how many of them will develop AIDS. Only prospective studies can serve.

Montagnier and the other members of a Franco-Belgian team have made an indirect and preliminary start on this part of the problem. Working in Zaire (once part of the Belgian Congo), they have traced the origin of the first AIDS cases presenting themselves at hospitals in Belgium in 1981. It now emerges (Brun-Vezinet *et al.*, *Science* 226, 453; 1984) that as much as five per cent of the population of Zaire may carry antibodies. By using sera kept in hospital refrigerators from earlier investigations, it has been possible to date one case of AIDS to 1977. The same team (Piot *et al.* *Lancet* ii, 65; 1984) has found unambiguous evidence of heterosexual transmission — and an annual incidence of AIDS as high as 20 per 100,000.

On the face of things, AIDS is nevertheless a new disease. Its nearly simultaneous appearance in Zaire, Haiti and the United States suggests that HTLV-III itself is a novel virus, presumably derived from something like HTLV-I. But how? And why does infection have a profound effect on only one class of T cells? Why should Kaposi's sarcoma, normally rare, be such a common sequel?

For the time being, these questions are wide open. But we are fortunate that Gallo and Montagnier were so quick off the mark, and that they and others now have the techniques they need. **John Maddox**

Fluid flow

Turbulence splits polymers

from A. Keller and J. A. Odell

EVEN minute quantities of long-chain molecules can dramatically influence the flow properties of liquids to which they are added, an effect that has many practical applications. However, limits are set on the usefulness and range of applications of polymer additives by the fact that the longest polymer chains are more effective but are also most likely to break during flow. Horn and Merrill¹ report on page 140 of this issue that such degradation of long flexible polymers subjected to turbulent flow conditions occurs by breakage at the midpoint of the molecules. Such apparently simple observations of degradation are also providing an insight into the molecular processes underlying many problems in fluid transport.

The countless examples of the use of polymers to modify fluid flow range from thixotropic ('non-drip') paints to engine oil viscosity enhancers. The energy required to pump liquids in turbulent flow can be reduced by up to 70 per cent ('drag-reduction') by the presence of a few parts per million of flexible long-chain molecules²; this has found practical applications in pipelines, heating systems and sewage disposal. Recently polymeric aircraft fuel additives have been developed to prevent fuel from 'misting', thereby dramatically reducing the risk of fire in the event of accident³.

The scientific approach to these effects has mostly been that of formal macroscopic hydrodynamics. Explanations on a molecular level have been proposed but are largely untested. A common hypothesis is that the effects are associated with the stretching out of flexible long-chain molecules in the course of flow. Since this process absorbs energy it could suppress droplet formation in misting, or defuse the nucleation of critical vortices during turbulent flow.

The earliest predecessors of Horn and Merrill's observation of midpoint scission of polymer molecules concern DNA⁴⁻⁶, which is not a highly flexible chain, but a rather rigid worm-like entity. Clearly a rigid rod-like molecule in any flow field will experience the greatest force at its centre, so that midpoint scission can be anticipated. But the more common flexible molecule chains are initially present as a tangle (random coil) and their scission would be expected to occur randomly along the chain. Only if the molecule is first straightened out can it be expected to behave as a rod and break at midpoint.

The systematic achievement of full stretching of chains through flow is by no means trivial, as conventional simple shear flows (such as laminar flow in a capillary or between rotating concentric cylinders)

merely distort the coil but do not straighten the chain. Instead, it is necessary to use special elongational flow systems in which there is a uniformly accelerating flow of very high velocity gradient (strain rate). Our own studies⁷ have shown that at a critical strain rate, which depends upon molecular length, the molecules undergo a sharp transition from the random coil to the fully extended state. At a higher and fixed strain rate, the increased force on the stretched-out molecules ruptures them at midpoint. The strain rate required to produce scission is proportional to the square of the length of the chain. This is predictable from the stresses arising in a stretched-out chain subjected to extensional flow. The deduced rupture strength of the chain is comparable to the strength of a covalent bond in the backbone.

This close correspondence between the fracture behaviour of coiled polymers under these model flow conditions and that of a straight molecule provides strong evidence that the coiled chains have been fully stretched out. Merrill and Horn, having corroborated these results and extended them to laminar flow through constrictions⁸, have now transferred the ideas and methodology to the less well

defined, but perhaps practically more important, condition of turbulent flow.

It has been hypothesized that in turbulent flows elongational flow fields prevail locally in vortices, producing local chain extension⁹. This cannot be observed directly on the microscale within the otherwise inhomogeneous turbulent flow, as could be done in the laminar model flow systems. Yet, as now reported, the unique effect of such flow and extension in the form of midpoint scission is observed⁸. By analogy to the experiments with model flow systems, Horn and Merrill infer that the chains must indeed have been stretched out by the turbulent flow field.

With the behaviour of macromolecules in turbulent flow now becoming accessible to experiment, we have reached a firm starting point for a molecular understanding of the intriguing and important flow-modifying effect of macromolecules and its consequences for fluid transport. □

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Molecular neurobiology

And now the sodium channel

from Charles F. Stevens

THE cloning and sequencing of the cDNA of the electric eel's sodium channel, described by M. Noda and seventeen colleagues on page 121 of this issue¹, reaffirms that S. Numa's laboratory at Kyoto University is the preeminent factory for cloning peptides and proteins of importance to neural function. More importantly, it is a key step in revealing the molecular basis of the electrical excitability of neural tissue and muscle.

What is the sodium channel and why is it important to clone and sequence its DNA? Channels — as the proteins responsible for brain electrical activity are called — fall into two nearly mutually exclusive classes: ligand-gated and voltage-gated. The former, when bound to by their ligands (specific neurotransmitter molecules) open to permit ion flux across the neuronal membrane. By contrast voltage-gated channels open and close in response to the voltage difference between the inside and outside of the neuron. The archetypal ligand-gated channel is the acetylcholine receptor (AChR). Numa's laboratory was the first to clone and sequence all four

subunits of AChR, and is now first to clone and sequence an example of a voltage-gated channel. In picking the sodium channel, they chose the membrane protein responsible for the nerve impulse.

Not only do AChR and sodium channels fall into different physiological classes, but they also belong to different structural classes. Whereas the AChR is a pentameric protein composed of structurally similar subunits, the picture of the sodium channel that emerges from the cDNA sequence of Noda *et al.* is of structurally similar domains within a single protein. The sodium channel of the electric eel is thought to consist of a single protein of >200,000 molecular mass. The cDNA sequence of this protein shows it to contain four domains, each of which is 250–300 amino acids in length and about 50 per cent homologous with the other three domains. Each domain has a similar overall structure with 6 identifiable subregions; two of these subregions are hydrophobic and presumably membrane-spanning, two are negatively charged, one has a large net positive charge, and one has no net charge.

The AChR is, cell biologically speaking, well behaved. To guide it through the endoplasmic reticulum during synthesis, it has an amino terminal signal sequence of the standard type for an integral membrane protein of eukaryotes. The sodium channel is more of a maverick in that it lacks an amino terminal signal sequence, as do a few other integral membrane proteins including ion pumps^{2,3}, the red cell chloride channel⁴, rhodopsin⁵ and the enzyme, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase⁶.

Where do we go from here? The first and most important step — one which will not be long in coming — must be to demonstrate that the entity whose sequence Numa's laboratory has deduced can, in fact, function as a sodium channel. This proof of function will come from expression experiments of the sort described by Miledi and Parker⁷: mRNA from the cloned cDNA, obtained either by transcription or hybrid selection, will be injected into frog oocytes, which will make the protein and insert it into their surface membranes. If the protein produced is really a whole sodium channel, it will be detectable by electrophysiological techniques.

Another high-priority experiment will be to obtain cDNA clones of other sodium channels, especially the mammalian one, perhaps through their cross-reactivity with the eel cDNA. One interesting strategy will be to look for mRNAs that code for unusually long strings of glutamates — the eel sodium channel cDNA has nine in a row, starting with residue 942. (This approach may also be a route to other voltage-gated channels and a way to start defining family relationships.) The extension from eel to other species is important both for the comparative information that will result, and because the function of the eel channel is hard to study with modern methods, whereas mammalian channels are better understood and more easily studied.

Some aspects of the structural features of the channel deduced by the Numa group are unattractive. For example, it is known that the coupling between membrane field and channel-gating is achieved through a change in the protein's dipole moment associated with the transition between open and closed forms of the channel. This dipole moment change is unusually large (hundreds of Debyes for the sodium channel as compared with tens for the AChR) and must reflect some unusual structural feature of the protein that places movable charges within the membrane, where they will feel the electric field.

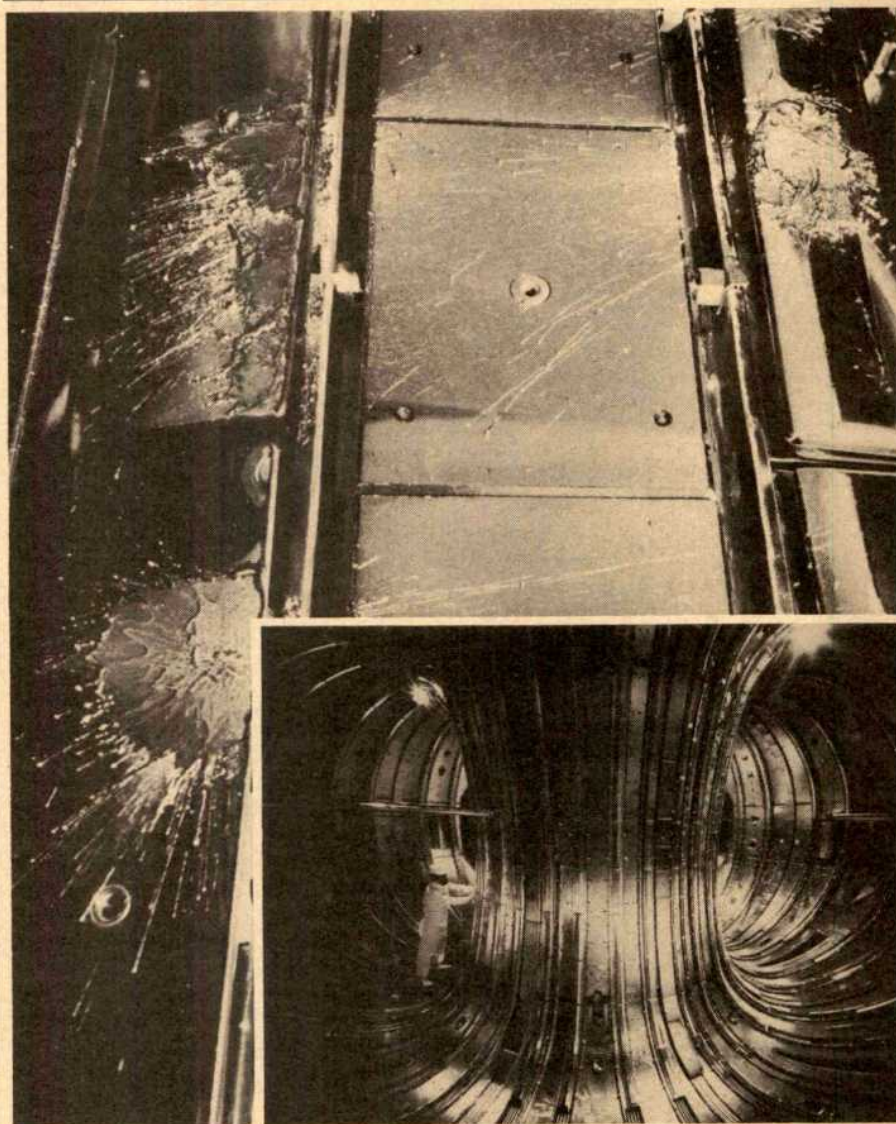
Unfortunately, no such feature is discernible in the structure suggested by Noda *et al.* As with the AChR channel, early efforts will be directed towards exploring alternative structures more in accord with physiological constraints.

Finally, we can look forward to structure/function studies of the channel, by means of site-directed mutagenesis of the cDNA. Numa's laboratory has a report in press in *Nature* using this approach for the AChR channel, and several other laboratories are carrying out similar experiments. The methods developed for the AChR will find quick application once clones for the sodium channel become more widely avail-

able. These are exciting times indeed for those with any interest in how the channels that control neural function operate. □

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Some 260 times since March, plasma pulses at the Joint European Torus (JET) in Culham have ended disruptively. The spectacular effect, shown above, is instantaneous melting of the Inconel alloy wall of the vacuum chamber by a beam of electrons stripped from the plasma during the disruption. The beam circles the chamber around half-a-million times in near-vertical magnetic fields, picking up energies of a few tens of MeV, and finally hits the inner wall to dump kilojoules of energy in a few milliseconds. The molten and evaporated metal can 'poison' carbon limiters used to limit the outer radius of the plasma ring in later pulses. The damage may contribute to JET's 'impurity problem', when chromium and nickel ions in the plasma radiate energy in interaction with plasma electrons, and limit the degree to which the plasma can be heated for a given rate of energy input. However, JET has shown a better ability to recover from disruptions — returning to normal performance within two pulses — than other tokamaks that have suffered from the same problem and have sometimes required a matter of days to recover.

Erratum

In Grant H. Thomson's article 'Details of Soviet cosmodrome from space shuttle photographs' (18 October, p.607), the words in italics in the following sentence were omitted: "Buildings and signs of construction activity in an area on the north side of the runway are thought to be preparations for the Soviet 'space plane'."

Active galaxies

Assembling the cloudy jigsaw

from Andrew Lawrence and Martin Ward

SOME galaxies have compact sources of radiation at their nuclei which display violent activity on a variety of timescales. It is difficult to identify a unique birthday for research on these active galactic nuclei (AGN), but there was no doubt that Professor Don Osterbrock of the Lick Observatory, and a distinguished AGN researcher, was 60 in July. At a workshop* held in celebration of that event, there was a feeling of substantial recent progress in three main areas: the understanding of the glowing gas surrounding AGN; the mapping of the overall energy budget of AGN at all emitting wavelengths; and the realization that activity in galactic nuclei is ubiquitous.

AGN are easily recognised by the bright optical emission lines in their spectra, arising from surrounding gas clouds heated by the central source of radiation. To understand the physical condition of the ionized gas, such as its temperature and density, much use is made of the relative intensities of the emission lines. D. Osterbrock reviewed the by now large data base on this topic, much of it gathered by himself and his graduate students at the Lick Observatory. This work has provided the information essential for the construction of models of line emitting regions. Indeed, a 'heavy industry' in AGN theory is that of modelling the shapes and strengths of the emission lines. Predictions from the standard model of a collection of identical optically thick clouds generally match the data well but several pieces of evidence imply the presence of a continuous range of densities and sizes in these clouds. In particular, B. Peterson (Ohio State University) argued from the variability of emission lines in AKN 120 that the clouds are uncomfortably close to the central radiation source and must evaporate, unless some of them are of a very high density. And W. Mathews (University of California, Santa Cruz) argued that symmetric Lyman alpha profiles further imply a substantial population of thin clouds.

Another outstanding problem is that of permitted ion emission, Fe II. This atomic species has an incredibly rich line spectrum in both the optical and the ultraviolet, but the spectrum is hard to model because, in certain parts, the multitude of Fe II lines can blend together to produce a false continuum (H. Netzer, Wise Observatory, Israel). In broad line radio galaxies, where the lines are even broader than usual, this may explain why Fe II was thought to be weak or absent. Since the lines are so broad they are 'washed out' and become part of

an apparently smooth continuum (B. Wills, University of Texas).

The profiles of these broad emission lines, produced by very fast cloud motions, must hold clues to the kinematics and geometry of the cloud regions. Much evidence points to radial motions but optically thick clouds, which would be bright on the side facing the central radiation source and dark on the back, should then lead to very different red and blue emission line wings. Because that is not usually observed, it further implies a mixture of thick and thin clouds. A. Filippenko (California Institute of Technology) and W. Ku (Columbia Astrophysics Laboratory) showed that lines from different atomic species often have very different profiles, again implying a physical stratification of the cloud regions. However, an interesting new idea regarding the line symmetry problem invoked the possibility of parabolic orbits, so that clouds are moving both out and in (J. Kwan, University of Massachusetts). And T. Kallman (Goddard Space Flight Center) suggested that in presence of a gaseous intercloud medium, scattering of ionizing photons could illuminate the back of clouds, making them bright on both sides. Finally, the radial motions need not be spherically symmetrical. M. de Robertis (Lick Observatory) presented a new model of a bi-conical expanding cloud region; viewed from different angles this could produce the various line profiles observed.

Energy budget

The second area of progress has been in understanding the continuum radiation from the central object itself. What is observed through a specific frequency window is often a mixture of components, such as synchrotron radiation, heated dust grain emission, atmospheres of hot young stars, and possibly thermal emission from a hot accretion disc surrounding a black hole. The sorting out of these different components, and their relative contributions to the total energy output of AGN, is now a major thrust of observational effort.

M. Malkan (Steward Observatory) argued for a 'thermal excess' in the blue over an underlying power law (the so-called 'blue bump') that may represent the radiation from an accretion disc. Others believe that this may be explained away by substantial Fe II and Balmer continuum emission from the surrounding clouds. M. Elvis (Smithsonian Center for Astrophysics) presented new soft X-ray data on optically selected quasars. The usually expected flattish X-ray slope was often not seen — rather, a variety of considerably steeper X-ray slopes were observed. Placed in the context of IR, optical and UV data, it

seems that the soft X rays in these cases may represent the high energy tail of the thermal excess postulated by Malkan. Furthermore a second 'little bump' is often visible on top of the 'big bump'. This presumably is the Fe II and Balmer continuum emission. This seems to resolve some of the controversy about the 'blue bump', in that we must be clear which 'bump' we are discussing.

J. Bechtold (Steward Observatory) and M. Malkan showed that the relative amounts of power-law and thermal components can be quite different in different AGN. Correlations within the properties of the power-law and thermal components, when considered separately, are generally good. M. Ward (Institute of Astronomy, Cambridge), using plots of the total energy output of AGN, emphasized that in many cases much of the energy must emerge at the peak of the thermal component in the extreme ultraviolet region (XUV). Unfortunately, this is currently an unobserved region — we see only the low energy UV head, and the high energy X-ray tail. To understand its true nature we need to observe the XUV body.

The third important area of progress is in recognizing that activity in the nuclei of galaxies is really widespread. As a result of sensitive spectroscopic surveys by W. Keel (Kitt Peak National Observatory) and T. Heckman (University of Maryland) it seems that 30 per cent of spiral galaxies have optical emission lines. This ionized gas cannot be produced by the radiation field of a population of normal semi-evolved stars. The required ionizing radiation could come from a small number of young, extremely hot massive stars, or be a dilute version of the power-law continuum radiation seen in quasars, or be shocked gas resulting from gas cloud collisions. Keel presented fresh evidence that these weakly active galaxies tend to have close companions somewhat more often than non-active galaxies — but the situation is confused. The presence of close companions seems to be a statistically significant factor, but is very far from being either a necessary or a sufficient condition for activity.

Much debate centred around the nature of the so-called Type II Seyferts. These are similar to the classical quasar-like AGN (most of which are classified as Type I Seyferts), but without the very broad emission lines or strong optical-UV-X-ray continuum. What remains are the strong narrow emission lines, and copious IR emission, almost certainly from heated dust grains. These are both features believed to come from the very large surrounding low density region. It is as if Type II Seyferts were Type I Seyferts with the energetic middle bits missing. D. Osterbrock and collaborators have long emphasized the existence of 'intermediate' types, with relatively weak broad lines and continuum, and evidence for a continuity of properties in several frequency regions was presented by A. Lawrence (Royal

*16-27 July 1984, Santa Cruz, California.

Greenwich Observatory) and R. Cohen (University of California, San Diego). However, A. Wilson (University of Maryland) argued that there exists a distinct difference in radio properties between Type I and Type II Seyferts. Some Type II Seyferts are really Type I Seyferts obscured by gas and dust; others may be 'switched off' Type I Seyferts — when the central radiation source switches off, the compact high density, high velocity cloud region will soon also disappear, but the very large low density regions may remain glowing for from hundreds to a few thousand years. Evidence for AGN changing their type on a timescale of decades was shown by Lawrence, Cohen and N. Bochkarev (University of California, Berkeley).

At least some of the classical Type II Seyferts may be quite different. J. Miller (Lick Observatory) reported that the Seyfert Type II NGC1068 has a highly polarised non-stellar continuum which extends through to the UV and soft X rays.

The emission lines are less polarised and those preferentially emitted from low density gas (the so-called 'forbidden lines') are polarised at a different angle from the permitted lines. This suggests that the central high density gas is present after all but for some reason is not moving at the great velocities characteristic of Type I Seyferts. G. Ferland (University of Kentucky) presented UV data for several Type II Seyferts which further showed that they do have a non-stellar continuum but that it is very weak.

Sitting through the workshop, one had the feeling of assembling a giant jigsaw puzzle. We have at least the majority of the pieces and have completed the edges. Now we must take a deep breath before attempting to make sense of the picture in the middle. □

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Neurobiology

Memory and molecular turnover

from Francis Crick

RECENT spectacular advances in molecular biology will, before long, begin to make a massive impact on certain key problems in neurobiology. A synapse is a complex piece of molecular apparatus, but there is now no technical reason why all its components — not merely the major ones — should not be isolated, sequenced and characterized. We can expect to have a detailed knowledge of the many ion channels, receptors and so forth, almost at will, for any type of neuron in any species. All this, together with techniques such as patch-clamping, should put the behaviour of synapses on a solid foundation and in the process tell us something about the molecular basis of memory. However, in spite of recent advances in understanding memory, in both *Aplysia* and long-term potentiation in the hippocampus, one aspect is often overlooked.

The time span of human memory (without obvious rehearsal) is often a matter of years, sometimes even tens of years. Yet it is believed that almost all the molecules in our bodies, with the exception of DNA, turn over in a matter of days, weeks or at the most a few months. How then is memory stored in the brain so that its trace is relatively immune to molecular turnover?

Several possible solutions of the problem suggest themselves. For example, memory might be coded in alterations to particular stretches of chromosomal DNA. Much current thinking assumes that memory is stored, at least in part, in the 'strength' of (many) individual synapses, so this would imply that in any neuron there is a special piece of DNA for each of its synapses, generated perhaps by similar means to

those used in cells of the immune system. This seems unlikely. A related alternative is that there is a special *local* piece of DNA (or perhaps RNA) for each relevant synapse. For example, it is conceivable that each spine apparatus has its own piece of nucleic acid which is modified when the system needs to alter the strength of that synapse. This idea also does not seem very likely but might be worth bearing in mind, since each mitochondrion has its own piece of DNA.

Another alternative is that each synapse has at least one macromolecule which is relatively immune from molecular turnover. Again this does not seem very probable but such a molecule could be looked for by appropriate pulse-chase experiments during development.

Since none of these alternatives seems especially attractive, one is more inclined to suggest models that are cooperative in nature. That is, the molecules in the synapse interact in such a way that they can be replaced with new material, one at a time, without altering the overall state of the structure. It is easily possible to conceive many such hypothetical models. It occurred to me to think of the simplest.

Consider, for example, a protein molecule that is an essential part of a synapse and can exist in two states: active and inactive. Suppose the molecule forms a dimer, probably with a two-fold rotation axis, that can be modified chemically by, for example, the attachment of a phosphate group. Let us assume that when both monomers are modified the protein is in the active state, symbolized by (+, +) and that when both are unmodified the dimer is in-

active, symbolized as (-, -). In addition, let us assume the existence of an enzyme that will add a phosphate group (or whatever the modifier is) to one monomer if the other monomer of the dimer is already modified but not otherwise. That is, it will turn a (+, -) into a (+, +) but will not touch a (-, -).

Assume, furthermore, that the mechanism for altering the synaptic strength can phosphorylate the dimer when more parts of the synapse are to be made active and can dephosphorylate it when activity is to be reduced. That is, the mechanism can turn a (+, +) into a (-, -), or vice versa.

Finally I assume that individual monomers of a single dimer can be replaced one at a time by the mechanisms responsible for molecular turnover and that these new monomers are initially unmodified.

This system will have the property that whether the dimer is in the active (+, +) state or in the inactive (-, -) state, the substitution of a single new unmodified monomer for either of its two components will leave it in the same state. That is, molecular turnover will turn a (+, +) into a (-, +) but the hypothetical enzyme will convert this back to (+, +). The (-, -) state will of course remain (-, -) during metabolic turnover. This mechanism is obviously modelled on known mechanisms for the maintenance methylation of DNA, as reviewed by Razin, A. and Friedman, J. in *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 25 (ed. Cohn, W.E.) 33 (Academic Press, 1981).

It is possible to imagine more complicated models of this general type. The macromolecule could be a trimer or a tetramer; there might be more than one modifiable site per monomer; rather than phosphorylation, the modification might be dephosphorylation, or methylation, glycosylation and so forth.

There are a number of subsidiary conditions. The macromolecule should be anchored to a particular synapse and not be free, in its mature form, to migrate from one synapse to another. This might suggest it would probably be a membrane protein and perhaps form part of a larger aggregate of some form. The supply of newly synthesized monomers should be immune to the modification (or demodification) process. The old monomers, due to be discarded by molecular turnover, should not interfere with the above processes.

Since the proposed mechanism is not implausible it would be sensible to look carefully for modifications to synaptic proteins and for the enzymes which modify and demodify them, in case one of these enzymes should have the peculiar characteristics described above. If such an enzyme were found it might prove to be a pointer to the seat of long-term memory. □

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Atmospheric carbon dioxide

Predicting plant productivity and water resources

from T.M.L. Wigley, K.R. Briffa and P.D. Jones

PLANTS love carbon dioxide. At elevated atmospheric CO_2 concentrations photosynthesis (and hence productivity) is enhanced, plants transpire less and make more efficient use of soil moisture reserves. In assessing the agricultural and hydrological consequences of anthropogenic CO_2 increases, we must, therefore, consider both CO_2 -induced changes in climate and the direct effects of increased CO_2 on plant physiology. The latter could be as important as the former, and may well be more easily predicted. Two recent papers have addressed different facets of this issue. In one, LaMarche *et al.*¹ suggest that recent increases in the growth of some high-altitude trees might be attributable to the direct effects of increased atmospheric CO_2 . In the other, Idso and Brazel² examine the effect of a CO_2 -induced increase in the efficiency of water use by plants on river basin evapotranspiration and river flow. Because Idso and Brazel assume that controlled-environment experimental results can be transposed to the real world, it is particularly important to demonstrate the validity of their assumption.

Before describing the work of LaMarche *et al.*, we can estimate the magnitude of increased tree growth that might be expected to occur under laboratory conditions due to a 25 per cent increase in CO_2 concentration, the change that has occurred since the mid-nineteenth century. To do this we use the well-known relationship between net primary productivity (NPP, roughly equivalent to tree ring width times mean density in this case) and CO_2 concentration (C): $\text{NPP} = \text{NPP}_0 [1 + \beta \ln(C/C_0)]$. β in this equation must be determined empirically and its value is still uncertain. Using $\beta = 0.3\text{--}0.7$ (see ref. 3) the implied change in NPP should be 7–16 per cent. This is a sufficiently small change that it would be exceedingly difficult to detect, given the natural variability of plant growth.

In fact, β may be even less. The values quoted are based largely on small-scale experiments which, as noted above, may not be applicable to the real world. A more relevant value can be estimated from carbon-cycle modelling studies but modelling uncertainties are such that a β value as small as zero is not ruled out. Since carbon-cycle models are our main tool for predicting future CO_2 levels, and since these predictions depend on the chosen value of β , it is clearly of importance to detect enhanced photosynthesis in the unmanaged biosphere in order to demonstrate that β is significantly greater than

zero.

In their field study, LaMarche *et al.* examined variations in bristlecone pine ring widths at two high-altitude sites in the White Mountains of eastern California, approximately 370 km east of San Francisco, and in limber pine ring widths at a site high up on Mount Jefferson, Nevada, about 180 km northeast of the other sites. At all three sites LaMarche *et al.* found an increase in tree growth since the early nineteenth century and particularly over the last 20 years. For the two Californian sites, where the tree ring data extend back many centuries⁴, the changes are unprecedented. Furthermore, the increases in ring width shown by LaMarche *et al.* exceed 70 per cent over the past 150 years, much greater than any anticipated changes (unless there has been a correspondingly large reduction in wood density). This clearly points towards some unusual cause, and CO_2 is implicated because it too has shown an unprecedented increase over the last century. However, if CO_2 were the cause we would expect the effects to be widespread and relatively small. Other bristlecone pine and limber pine chronologies exist near to the sites studied⁴, but not all of these show the same upward growth trends. Further research is required to explain this site specificity and the apparently large amplification of the CO_2 effect. Could, for example, an amplified effect occur specifically at locations near the tree line if there were a CO_2 -induced reduction in the threshold temperature for photosynthesis?

Alternatively, are these changes due to climatic change? LaMarche *et al.* consider only temperature and precipitation changes (neither show any recent trends), and their analysis is inconclusive because the climate–tree growth relationship is not well-defined. To complicate the issue further, climate may be indirectly involved; for example, since 1960 the regional westerly wind component has increased in a way that, at least superficially, matches the tree growth trends. Could changes in nutrient input due to pollutant transport from the industrial and agricultural areas around San Francisco be the link? While intriguing, the data of LaMarche *et al.* are not yet enough to support any claim that the growth changes are related to CO_2 increases, and the authors are justifiably cautious in their conclusions.

Idso and Brazel are not concerned with small past changes, but with the much larger increases in CO_2 that might occur in the future. They consider the case where the CO_2 level is double the pre-industrial

level — a value expected to occur around the mid-twenty-first century — and examine the effects of CO_2 -induced changes in water use by plants in line with the NPP relationship given above.

Higher CO_2 levels reduce evapotranspiration by causing the stomata to close. When evapotranspiration is reduced, more precipitation will be available for runoff, so reducing the normal depletion of soil moisture reserves during the growing season. Thus, whereas the potential effects of any reduction in precipitation caused by increased CO_2 concentration could be offset via the direct effects of CO_2 on evapotranspiration, the effects of an increase in precipitation might be enhanced. To study the relative effects of these two processes requires both a knowledge of the future change in climate and a detailed model of the vegetation. Idso and Brazel use a simple water balance calculation, coupled with the climate scenarios assumed in an earlier study⁵, to assess the sensitivity of runoff to both effects. Their results indicate that direct CO_2 effects could more than offset influence of even such large changes in climate as a 10 per cent drop in precipitation coupled with a 2°C rise in temperature. In a much more complex analysis, Aston⁶ has reached a similar conclusion.

The changes in agricultural and hydrological resources predicted by these studies will have to be considered carefully in future planning. Both, however, are exploratory, and depend on a number of simplifying assumptions. Idso and Brazel, for example, take no account of the seasonal distribution of precipitation and evapotranspiration. If most of the runoff were associated with winter precipitation (for example, through snow melt) then a reduction in drainage basin evaporative losses during the growing season could have only a minor effect on annual runoff. Even during the growing season, the reduction in evapotranspiration may be offset by an increase in leaf (transpiring surface) area, a factor considered in neither study. Calculations by Goudriaan *et al.*⁷ using a very detailed plant physiological model, show that increased leaf area could largely compensate for reduced transpiration rate per unit leaf area. However, as Aston points out, this would not operate in tropical regions where C-4 plants often dominate.

These are all exceedingly complex issues. Future changes in atmospheric CO_2 level

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are expected to far exceed any changes that have occurred to date. Even in the absence of marked changes in climate, atmospheric CO₂ may have dramatic effects on agriculture and water resources. To support rational planning for this eventuality, we require more studies like those discussed here to increase our quantitative knowledge of the influences of CO₂. But

much more groundwork is required before we can make predictions that have practical value. We may have only a decade or so in which to make the step from speculation to application. □

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Palaeoecology

Hampstead Heath clue to historical decline of elms

from Peter D. Moore

LIKE human history, palaeoecology can be a frustrating occupation. How can one hope to construct a satisfactory, scientific explanation for an event which appears to have taken place on only one occasion in the fossil record? The whole basis of uniformitarianism begins to totter at this point. On the other hand, such events can fuel endless speculation, the pleasures of which tend to offset the frustrations. One of these has become a recurrent theme in the Quaternary palaeoecology of Europe: the decline in elm pollen which occurred quite abruptly across much of northern Europe, some five thousand years ago. It was this theme which reared its enigmatic canopy over a recent conference of botanists and archaeologists at Oxford*.

In the past few years, the debate has lost something of its vigour simply because no new evidence has come to light. It has been established that the collapse of the elm pollen in the stratigraphic record is widespread, relatively synchronous (within a 300–400 year period over a very large area, as indicated by dates which themselves, however, have broad inherent error margins). There are some isolated locations where the elm decline is less marked, but even in the extreme north of Europe, far beyond the limits of elm, it is often possible to detect a drop in the elm pollen that had been transported over considerable distances.

One of the major controversies has centred around the coincidence of the first clear indications of agricultural activity by Neolithic peoples with the elm decline, which has led to interesting speculations about primitive forestry practice and contemporaneous human population levels. Models advanced to explain the elm decline have evolved over the decades in a punctuated manner, each quantum leap being dependent on the acquisition of some new item of circumstantial evidence which permits further elaboration, or even a complete restructuring of the theory. Climatic change gave way to soil deterioration, which was overthrown by the radical

proposition that man stripped the elm trees of their branches in order to feed stockaded domestic herbivores.

After a period of equilibrium, if not stagnation, the topic seems set to make another leap, and the evidence is coming from a number of directions. In the first place, it has become apparent that agricultural activity in Britain was widespread prior to the elm decline. This fact was demonstrated at the Conference by the data of J. Innes and I.G. Simmons (University of Durham). Their work on the North York Moors clearly shows that there were episodes of forest clearance during what are presumed to be Mesolithic times. Evidently the advent of agricultural ideas and practices in northern Europe was not a sudden, revolutionary event. In a recent analysis of the first occurrence of cereal-type pollen grains, Edwards and Hiron (*J. archaeol. Sci.* 11, 71; 1984) showed a wide geographical scatter of records in Britain prior to the elm decline. Whether these derive from Mesolithic or Neolithic cultural activity is impossible to say and may in any case be a rather meaningless question. What is important is that the earliest agriculture was underway before the elm decline, which was therefore not the outcome of any large-scale invasion of innovative, agricultural techniques.

A similar conclusion has been drawn by J. Turner (University of Durham) who displayed pollen diagrams based on contiguous 1 mm microtome slices of a frozen peat core from the North Pennines. These showed a progressive decline in elm, evidently related to the succession of clearance events which had been taking place in the region over a period of several hundred, possibly a thousand years. Since the elm decline is also accompanied by falling levels of lime (*Tilia*) and ash (*Fraxinus*), Turner is inclined to the idea that the cumulative effect of centuries of Mesolithic wear and tear on the vegetation has resulted in the depletion of soil nutrients, leading to a decline in all three species. She has also proposed, on the basis of the pore numbers in the fossil elm pollen, that the elm in question in the area was not the wych elm, *Ulmus glabra*, as

expected, but was more probably *U. minor* (see Stockmarr, *Danm. Geol. Unders.* IV R 4, No. 11; 1970). Since this taxon is climatically more sensitive than the wych elm, the suggestion could cause the entire debate to return to its starting point of some forty years ago.

It was at this point, however, that fuel was added to the fire from an unexpected quarter. M. Girling (English Heritage) announced the discovery of wing cases from an interesting beetle in sediments just 10 cm below the elm decline at a site on Hampstead Heath, London. The beetle she identifies as *Scolytus scolytus*, the carrier of the fungus, *Ceratocystis ulmi*, that is the cause of Dutch elm disease.

The proposal that the decline of elm 5,000 years ago was the product of a disease is not in itself new (see W.A. Watts, *Proc. Linn. Soc. Lond.* 172, 33; 1960). It has become increasingly attractive, even compulsive, as we have observed the recent effects of Dutch elm disease on elm populations. But healthy speculation feeds upon circumstantial evidence and here, at last, we seem to have it. If the beetle vector was here 5,000 years ago, perhaps the disease was too. Was the sudden onset of the disease the result of early forestry? And how do we explain the concurrent decline in some other trees, such as lime? The answers are still not clear, and can never be entirely testable, but the new evidence will provide the necessary momentum for renewed vigour in an old debate. □

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100 years ago

NATURAL SCIENCE IN SCHOOLS

HOWEVER fully it may be admitted by the few that it is important, nay essential, that all members of the community, whatever their station or occupation should during their school career receive some instruction in the elements of natural science, the general public have not as yet had brought home to them with sufficient clearness that, just as a knowledge of foreign languages is essential to all who are brought into intercourse with foreigners, so in like manner is a correct knowledge of the elements of natural science of direct practical value to all in their daily intercourse with Nature, apart from the pleasure which such knowledge affords. In fact, from a purely utilitarian standpoint, the advantages to be derived from even most elementary acquaintance with what may be termed the science of daily life are so manifold that, if once understood by the public, the claims of science to a place in the ordinary school course must meet with universal recognition. To quote Huxley: "Knowledge of Nature is the guide of practical conduct. Any one who tries to live without attention to the laws of Nature will live but a very short time."

*Joint meeting of the Botanical Society of the British Isles and the Association for Environmental Archaeology, Oxford, 21–23 September, 1984.

Psychiatry

Complexities of cause and effect in anorexia nervosa

from Nicholas Mrosovsky

EXTREMES in body weight such as gross obesity or the emaciation of anorexia nervosa are accompanied by numerous physiological, behavioural and psychological changes. These disturbances are so interwoven that it is difficult to disentangle the primary factors. Classic studies by Sims and Horton¹ have demonstrated that, simply by overeating, volunteers of normal weight can obtain obese-type endocrinological profiles; when the period of overeating ends, body weight returns to normal and the endocrinological symptoms subside. Such findings suggest that, at least in some types of obesity, these particular symptoms are a consequence of being fat rather than instrumental in initiating overweight. This approach has now been applied to the problem of anorexia nervosa by Fichter and Pirke and their co-workers at the University of Munich and the Max-Planck-Institut für Psychiatrie².

The well-known Minnesota studies of Keys *et al.*³ have already shown that starvation induces a number of features that are common in anorexia nervosa; obsession with food and rituals surrounding meals are obvious examples. However, these observations were made on young men, while Fichter and Pirke selected subjects and measures with anorexia nervosa specifically in mind. Young women in their early twenties and close to 'ideal' weight volunteered to go without food, other than certain minerals and vitamins, for three weeks. A number of changes, reminiscent of anorexia nervosa, occurred during the fast, including elevated blood cortisol, abnormal dexamethasone suppression tests, blunted response to thyrotropin-releasing hormone, lower levels of luteinizing hormone (LH) and regression of the 24 h LH secretory profile to the infantile pattern. The LH changes were not consistent. However, the average weight loss of 8 Kg was small compared with percentage reductions from ideal body weight commonly observed in anorexia nervosa. In general, apart from some less clear data with growth hormone, "the results support the hypothesis that the endocrine disturbances reported in anorexia nervosa are not primary but rather the consequences of reduced food intake or loss of body weight"².

While the results of the Fichter and Pirke study are consistent with the view that many physiological symptoms of anorexia nervosa are secondary to weight loss, there remain counter-arguments. The relationships of endocrine function to body weight in anorexics are by no means simple. For instance, weight recovery does not always

lead to normalization of LH levels⁵ and changes in the metabolism of brain nor-epinephrine sometimes last for months after nutritional rehabilitation⁵.

An abiding question about such counter-arguments is what do we mean by 'recovery'? Usually this is defined in terms of attaining a weight near to the ideal level for the age and height of the patient. This is far from satisfactory since it is not uncommon for anorexics to have been above average weight prior to the disease. Persistence of endocrinological abnormality in an individual might reflect a failure to recover to an appropriate weight for that individual. What is needed are indices of recovery other than average population weights from the Metropolitan Life Insurance tables. Even when the patient's own pre-morbid weight is used, there is the problem of defining the onset of the disease; moreover, anorexics often have unstable weights. Further studies of the effects of fasting and refeeding on people of stable weight, who do not normally diet, should provide better indices of deviations from an individual's own physiologically-regulated weight level. Without these, the relationship of endocrine function to body weight in anorexic patients may remain obscure.

The idea of a strong biological component in anorexia nervosa has been entertained ever since the disorder was first described. Prospective longitudinal studies, with measurement of many physiological variable are costly and difficult. Given this vacuum, the experimental approach taken by the Munich group provides some of the most important information we have on the topic and may be destined to take its place alongside other classic studies on weight manipulation in normal subjects. Of course, these studies do not preclude predisposing factors or the possibility that a primary abnormality in metabolic regulation may result in weight changes which in turn produce secondary changes¹. Therefore the question of whether there are primary physiological disturbances in anorexia nervosa has not yet been resolved. It would be a fair summary of present knowledge to say that not one of the somatic symptoms has yet been proven to be primary in the disorder, and that a large number of these symptoms are not specific to anorexia nervosa, but can be produced by weight loss alone. □

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Nuclear physics

From little bang to Big Bang

from Neil Rowley

AT sufficiently high incident energies, the system formed in a head-on collision of two heavy nuclei will 'explode', showering fragments in all directions. A new generation of particle detectors, which almost completely surround the target in such a collision, makes it possible to detect enough members of the shower to reconstruct some details of the initial event. Two experiments of this kind have recently been completed on the Bevalac at the Lawrence Berkeley Laboratory in California; the results of both hint at the possibility of some exciting new physics.

The first experiment, by H.A. Gustafsson *et al.* (*Phys. Rev. Lett.* **52**, 1590; 1984) employed the 'Plastic Ball' spectrometer to investigate the possibility of observing collective flow of nuclear matter in Ca + Ca and Nb + Nb collisions at 400 MeV per nucleon. The observation of such collective effects would give hope that some very exotic phases of nuclear matter could be created by relativistic

heavy-ion collisions.

In order to explore an equation of state and look for possible phase transitions, a large sample of matter needs to be taken and subjected to controlled variations of temperature and pressure. This is not so easy for nuclear matter since we only possess relatively small samples — the stable nuclei — and we can only vary the ambient conditions by forming hot compressed systems in short-lived nuclear reactions. Here the finite size of the nucleus is an apparent problem since nucleons with a high kinetic energy might rapidly escape from the compound system and thermal equilibrium may never be established.

At low excitation energies, the mean-free-path of a neutron or proton in a heavy nucleus is indeed large (at least when compared with the nuclear radius), though each nucleon is confined by the average field of all the others. Thus one might expect that highly excited nucleons would escape without scattering. The reason for

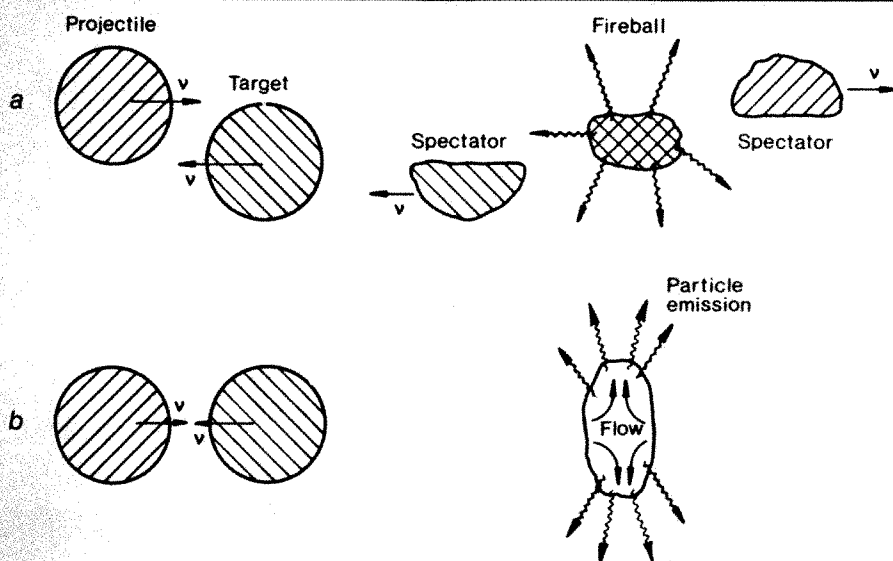


Fig. 1 High-energy collision of identical nuclei in the centre-of-mass frame. *a*, Non-central collisions. The sections of target and projectile which did not overlap remain as relatively 'cold spectators' moving with their original velocities. The overlapping sections form a 'fireball' whose centre-of-mass is at rest. There may, however, be collective flow in the fireball which decays rapidly by thermal particle emission. The final momentum distribution has the collective momenta superimposed on top of a statistical distribution from the decay. *b*, Head-on collision. All the mass is compressed into the fireball. In the hydrodynamical model this mass is squeezed out in a collective flow at right angles to the original beam direction. The system still decays by statistical emission but with an additional perpendicular component to the momentum. The flow may be very different if the target and projectile have significantly different masses (see text).

the large mean-free-path is, however, rather special. Nucleon-nucleon interactions are inhibited by the Pauli principle which prevents a neutron (proton) from scattering into a state already occupied by another neutron (proton). For very fast collisions many high-energy unoccupied states become accessible, the nucleon-nucleon scattering regains its full vitality and the nucleon mean-free-path becomes very small. At 400 MeV per nucleon the Pauli blocking may essentially be ignored.

It has already been established that in high-energy nucleus-nucleus collisions the emitted charged particles have an energy distribution which is consistent with a system that has fleetingly achieved thermal equilibrium. The observation of equilibrated particle spectra unfortunately tells us nothing about the internal pressure of the system. At the limit of small mean-free-path, however, a system should behave hydro-dynamically and it should be possible to set up local pressure gradients and generate collective flows. The problem is that long before reaching any detector, the hot 'fireball' formed from the overlapping regions of the target and projectile (see Fig. 1) will have 'radiated' away to nothing. The only way of knowing if such hydrodynamical effects exist is to detect as many as possible of the radiated particles, measure their momenta and reconstruct the flows existing before the fireball disintegrated. This was the aim of the experiment Gustafsson *et al.*

A further difficulty in interpreting the data from such experiments is that hydro-dynamics predicts collective flows which depend on whether the collision is head-on

(see Fig. 1*b*) or more peripheral (Fig. 1*a*). The enormous power of a detector like the Plastic Ball lies in the fact that it is actually a large number (815) of electronically coupled detectors covering a solid angle of almost 4π , that is almost completely surrounding the target. By detecting many low-mass charged particles in coincidence with one another, it is possible to have a high degree of confidence that they originated from a single event and to relate qualitatively the particle multiplicity to the size of the fireball.

For identical nuclei the most dramatic event is the head-on collision — giving rise to high multiplicity. Here the hydro-dynamical model predicts that the matter is squeezed out at right angles to the original beam velocity (see Fig. 1*b*). This event is referred to as 'sidesplash' and the direction of flow relative to the beam axis is specified by the flow angle. This should be 90° for a head-on collision. For the Ca + Ca experiment of Gustafsson *et al.* the results are disappointing. For all but the highest multiplicities the particle cross section is peaked at 0° , that is along the axis. This is consistent with a purely statistical decay and gives no evidence for collective flow. Only for the highest multiplicity events is a small deflection, of around 10° , observed.

For the more massive Nb + Nb experiment the results are much more encouraging. A finite flow angle is observed and it increases steadily with increasing multiplicity up to a maximum value of around 30° . This is far short of the expected 90° for a head-on collision but the occurrence of such a direct event is rather infrequent and the flow angle drops rapidly

to zero with decreasing projectile-target overlap. The theoretical analysis of Buchwald *et al.* (*Phys. Rev. Lett.* **52**, 1594; 1984) shows that when these effects and the detector efficiencies are taken into account a maximum average flow angle of about 30° means that collective hydrodynamical flow has been established.

More recently the mass-asymmetric system Ar + Pb has also been studied at the Bevalac in collisions at energies around 800 MeV per nucleon (Renfordt, R.E. *et al.* *Phys. Rev. Lett.* **53**, 763; 1984). Here an interesting twist to the flow-angle behaviour has been observed: for very central collisions (highest multiplicities) the fragment distribution is almost isotropic and there is no discernible flow angle.

Surprisingly, this result is again consistent with the hydrodynamical model which shows that the behaviour is due to the different 'geometry' of the system. In a central collision the Ar nucleus is totally engulfed by the larger Pb target in which it is brought to an abrupt halt. Since the kinetic energy is entirely deposited in the interior of the target this results in an almost isotropic expansion of the composite system. Statistical or 'cascade' models (see Cugnon, J. *et al.* *Phys. Lett.* **109B**, 167; 1982) do not produce such a large stopping power since they cannot describe the vital compressional modes. In these models some remnant of the initial beam velocity survives giving a forward peaking of the fragment distribution — a prediction inconsistent with the results of the second experiment and the Nb + Nb data.

For lower multiplicity events, however, the asymmetric system does exhibit a finite flow angle. The maximum value appears to occur for collisions where the Ar projectile is just eclipsed by the larger Pb target. This configuration now resembles the head-on collision for the mass-symmetric system, since the non-overlapping part of the target may be regarded as a spectator. The result is again elegantly reproduced in the hydro-dynamical picture.

It does therefore seem possible to compress nuclear matter in such experiments. If compressions to around three times normal density can be achieved, then theory predicts the existence of a nuclear phase containing a Bose-condensate of pions. An even more alluring goal (though higher energy beams would be required) is the creation of a fireball in the phase of a quark-gluon plasma. Such experiments are our only possible means of reproducing in the laboratory a state of matter that only existed at the very beginning of the Universe. The creation of such exotic phases may still be far away but the new Bevalac results are an important step in the right direction. □

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Molecular evolution

On the origin of the *Alu* family of repeated sequences

from Andrew Leigh Brown

7SL RNA is an example of a eukaryotic RNA species that was identified long before a cellular role could be ascribed to it. Only recently was it established¹ that it forms an essential part of the signal recognition particle — the cellular apparatus that aids the transport of secreted proteins across the endoplasmic reticulum. Subsequently nucleotide sequence analysis revealed that parts of the 7SL RNA sequence seem to derive from the *Alu* element², which is repeated about 300,000 times in the human genome³. While *Alu* is usually found as a head-to-tail dimer of a sequence about 150 base pairs long, in the 7SL sequence a single *Alu* monomer seems to have suffered an insertion of 155 base pairs. This was not in itself a dramatic discovery because *Alu* elements are dispersed so widely throughout the human genome that they could easily be a target for random DNA insertion. However, the latest developments concerning *Alu* and 7SL RNA^{4,5}, some of which are published on page 171 of this issue, have taken a quite unexpected turn.

Ullu and Tschudi⁴ have sequenced 7SL RNA (as a DNA clone) from *Xenopus* and *Drosophila*. Both are clearly related to the human counterpart throughout their length — that is the sequence homology extends to the *Alu*-like part of the human molecule. The presence of an *Alu*-like sequence in *Drosophila*, also found by Gundelfinger *et al.*⁵, means that *Alu* has had a much longer evolutionary history than was previously thought. More interesting yet, in *Drosophila* it seems only to exist as part of the 7SL DNA, of which there are two copies. On this basis, Ullu and Tschudi suggest that *Alu* sequences represent defective 7SL RNA molecules that have been reverse-transcribed into DNA and inserted into the genome. An analogous origin has been suggested for alpha-globin pseudogenes in the mouse⁶, and the multiple pseudogenes for small nuclear RNAs in man⁷. Pseudogenes are generally thought not to play an important role in the cell. Perhaps those who have argued that *Alu*, by its very abundance, must have an important function will recognize that this argument has now lost some of its weight.

Although it seems likely that the 7SL sequence with its single *Alu* evolved in the absence of repeated *Alu* elements, an alternative interpretation of Ullu and Tschudi's data presents itself, at first sight. In the 7SL-specific region of the sequences the match between the human and *Drosophila* sequences is 67 per cent, and the correct alignment is obvious. In the *Alu*-related re-

gions the degree of match is less and the two groups opt for rather different alignments, especially at the 3' end. If, in fact, these regions of the sequence are completely unrelated, then the earlier hypotheses could still be tenable. However, using the method of Fitch and Smith⁸, I find that the alignment of the *Drosophila* sequence with the 3' *Alu*-related part of the human 7SL sequence suggested by Ullu and Tschudi has a probability of less than 0.01 of occurring by chance. Thus the flanking regions of the *Drosophila* 7SL RNA are homologous to *Alu* in man and the *Alu* sequence first existed as a part of this molecule.

As expected, for *Xenopus laevis* the overall match with the human sequence is much higher⁵ than either is with *Drosophila*, although it drops off in 3' *Alu*-related region. Although their previous results indicate only weak homology to *Alu* in *Xenopus* DNA⁹, Ullu and Tschudi describe the *Xenopus* genome as being 'rich' in sequences related to the *Alu*-like parts of the *Xenopus* 7SL molecule. So, in *Xenopus* as well as man, there is a close relationship between the 7SL RNA and highly repetitive elements, although the *Xenopus* and human sequences have diverged during evolution. How did this relationship arise? The fact that 7SL has a longer evolutionary history, being found in the absence of any *Alu*-related repeats in *Drosophila*, strongly suggests it was the antecedent. Moreover, the 7SL RNA is arranged in the signal recognition particle in such a way that three RNA fragments similar to the 5' *Alu*-related, 7SL-specific and 3' *Alu*-related sequence domains are generated by limited enzymatic digestion of the intact particle¹⁰. Put that together with the circumstance in which the 7SL RNA was first discovered — in a retroviral particle¹¹ and therefore close to a source of reverse transcription activity — and the generation of DNA copies of *Alu* is easy to imagine.

Amplification

Once *Alu* had appeared, what happened next? It has been known for some time that *Alu* is a remarkably stable component of the genome. Having arisen at a specific location, it does not seem to disappear quickly. For example, in the gamma-globin region, two *Alu* elements are present in exactly the same place in both man and chimpanzee¹². This is in striking contrast to the vagility shown by the middle repetitive DNA elements in *Drosophila*, and until now there has been no real explanation for the difference. However, if *Alu* originated

as a 7SL pseudogene it may not possess or respond to any mechanism for excision or transposition. Thus, unless it kills the chromosome in which it inserts, it might just have to stay there. If it retains polymerase III promoter activity (not all copies do retain it), it could even generate more transcripts, each of which might be another possible template for reverse transcription and reinsertion. One would therefore expect copies to accumulate in the genome.

Two questions are left. First, did the events which gave rise to the *Alu*-related repetitive elements occur once in each of the *Xenopus* and human lineages independently? If so, it might explain the sequence divergence between repeats of the two species; within the species the repeat sequence very closely resembles the 7SL sequence. However, we should remember that some concerted evolution has probably taken place. It is possible to envisage repeats already existing in the primitive amphibian DNA and gradually, after the two lineages separated, changing *en bloc*. Any subsequent generation of pseudogenes would be of the species-specific 7SL sequence; a process of concerted evolution which kept the 7SL and *Alu*-related repeats reasonably similar within each species is not unlikely.

The second question is, why has none of this occurred in *Drosophila*? Here there are no easy answers. The situation is similar to the case of the small nuclear RNAs, where many pseudogenes are found in man⁷, but not in *Drosophila*¹³. Processed pseudogenes of protein coding regions also seem to be absent in the fly. There is certainly no absence of reverse transcriptase activity which might explain this — generation of reverse transcripts may be a major mechanism for replication of *copia*¹⁴. Nevertheless, reverse transcripts do not accumulate in the genome of *Drosophila* in the way they have in vertebrates. When we know why this is so, we shall be well on the way to understanding why the genomes of insects are so very much smaller than those of vertebrates despite containing much the same amount of information. □

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Towards a model for the arms race

SIR — Saperstein's use of a discrete recursion model to describe the arms race¹ is intriguing. He uses equations derived from the study of chaotic behaviour, as manifested in fluid dynamics. We have two theoretical objections, but in addition, his model's predictions do not agree well with the available data.

The model states:

$$x_{n+1} = 4ay_n(1 - y_n) \quad (1)$$

and

$$y_{n+1} = 4bx_n(1 - x_n) \quad (2)$$

where y_n represents the ratio of arms expenditure to Gross National Product (GNP) in country A (say the Soviet Union) in the year n , and x_{n+1} represents the same ratio for country B (say the United States) in the following year. The stability of the arms race is predicted from the disposable parameters a and b .

According to these equations, as the expenditures of nation A approach its GNP, those of nation B approach zero. This does not seem reasonable. A kind of saturation term might be preferable, but would probably not produce the "chaotic" behaviour desired.

Although the ideas are linked semantically, the chaos of warfare and of fluid dynamics are otherwise dissimilar. In warfare, the variables representing arms expenditures do not undergo a transition to a wandering unpredictable distribution, as the equations imply. Predictably, they will show a dramatic increase.

In his examples of various arms races, Saperstein gives data for only two years. For the Soviet Union and the United States, data are available² for the 10-year span 1971 to 1980 (see table below). To compute a least squares estimate for the parameter b requires minimizing the function

$$f(4b) = \sum_{i=1}^{n-1} [4bx_i(1 - x_i) - y_{i+1}] \quad (3)$$

Setting $f'(4b) = 0$, the function has a minimum at

$$4b = \frac{\sum_{i=1}^{n-1} [x_i(1 - x_i)(y_{i+1})]}{\sum_{i=1}^{n-1} [x_i(1 - x_i)]^2} \quad (4)$$

This gives $4b = 2.55$, or $b = 0.64$ (compared with 0.54 as estimated by Saperstein.) Similarly, $4a = 0.467$, or $a = 0.12$ (compared with 0.15). After transforming

the variables in the nonlinear equations, linear regression can be performed:

$$y_{i+1} = 4bX_i, \text{ where } X_i = x_i(1 - x_i) \quad (5)$$

and

$$x_{i+1} = 4aY_i, \text{ where } Y_i = y_i(1 - y_i) \quad (6)$$

Equation (5) represents the prediction of Soviet expenditures based on previous US expenditures, and equation (6) the reverse.

The correlation coefficient measuring agreement between the model and the actual data is given by

$$r^2 = SS_{\text{regression}} / (SS_{\text{regression}} + SS_{\text{residuals}}) \quad (7)$$

where

$$SS_{\text{regression}} = \sum_{i=1}^{n-1} (4bX_i - \bar{y})^2, \quad (8)$$

$$\bar{y} = 1/(n-1) \sum_{i=2}^n (y_i) \quad (9)$$

and

$$SS_{\text{residuals}} = \sum_{i=1}^{n-1} (y_{i+1} - 4bX_i)^2 \quad (10)$$

For the prediction of Soviet expenditures from those of the United States, $r^2 = 0.518$. Analogously, the prediction of US expenditures from those of the Soviet Union gives $r^2 = 0.130$. A plot of the residuals demonstrates a clear trend, indicating a systematic error in the model.

For comparison, Hamblin³ obtains correlation coefficients better than 0.9 in applying his curvilinear model to arms expenditures before the First World War, to "warlike worktime" before the Second World War and to satellite launchings, ballistic missile production and nuclear explosions in more recent times. These data sets are more accurate than the proportion of GNP devoted to arms, which rests on many assumptions. The asymmetry of Hamblin's model (in contrast to Saperstein's) has serious policy implications: he assumes one party to the arms race to be the leader.

While Hamblin³ and Richardson⁴ discuss the possible meaning of their parameters, Saperstein unfortunately does not speculate on what might influence the values of a and b , and hence the stability of the arms race. We share his concern that we may be closer to the threshold of war than the model suggests, especially considering the magnitude of actual expenditures rather than the theoretical parameters. The proportion of resources devoted by the

Soviet Union to arms has been matched in peacetime only by that of Nazi Germany just before the Second World War.

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Basement membranes and epithelia

SIR — There is considerable confusion over the nomenclature of "basement membranes" of epithelia. We propose, in accordance with usage in a number of leading laboratories, to restrict the term *basement membrane* to the (usually) fibrillar layer produced by the connective tissue fibroblasts, and to use the term *basal lamina* only for the acellular glycocalyx immediately underlying the epithelial cells. The basement membrane can always be resolved by light microscopy, the basal lamina as a rule only by electron microscopy. The distinction is a morphological and operational one and as such is perfectly unambiguous. (We leave aside the question whether the basal lamina may be regarded as part of the basement membrane.)

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Gene conversions and crossing-over

SIR — Fink and Petes¹, in their News and Views commentary on the interesting papers by Klein² and by Klar and Strathern³, refer repeatedly to the "50 per cent rule" relating gene conversion to crossing over and state that this rule has been so compelling as to have been "incorporated into virtually every current model of recombination". This may give a wrong impression. As Klein points out, it has long been known that intragenic recombination at meiosis within certain genes in various eukaryotes may involve crossing over far less than 50 per cent of the time. An association of as low as 10 to 30 per cent is not at all unusual in *Sordaria*⁴, *Neurospora*⁵ and *Drosophila*⁶. Even in *Saccharomyces*, as Stadler pointed out in his 1973 review⁷, the figure comes down to 35 to 40 per cent when proper allowance is made for coincidental crossovers.

It would be remarkable if virtually every current model really were committed to a 50 per cent rule, and in fact this is not so.

Proportion of GNP devoted to arms expenditure

Year	United States	Soviet Union	Year	United States	Soviet Union
	x_i	y_i		x_i	y_i
1971	0.070	0.144	1976	0.053	0.134
1972	0.066	0.147	1977	0.053	0.131
1973	0.060	0.142	1978	0.051	0.142
1974	0.061	0.142	1979	0.051	0.143
1975	0.059	0.144	1980	0.055	0.146

The model proposed in 1975 by Meselson and Radding⁸, which was put forward for quite another good reason (the need to reconcile the Holliday model with the increasingly strong evidence for hybrid DNA on one participating chromatid only), provides a very plausible way of uncoupling conversion from crossing-over to any desired extent. According to this model, the first step leading to gene conversion is the unilateral invasion of one participating chromatid (DNA duplex) by a single DNA strand unwound from the other. The reciprocal strand-crossing and the formation of the symmetrical cross-strand structure (Holliday junction), which hypothetically sets up the possibility of crossing over, follows later after a series of manoeuvres that could easily require special conditions (such as may prevail more or less during meiosis) for their efficient performance. An apparently easy alternative is for the initial single bridge to be cut by an endonuclease, the invading single-strand fragment ligated into the recipient chromatid and the gap in the donor chromatid filled by repair synthesis. This could lead to conversion without any possibility of crossing over.

Since crossing over (both in fact and in theory) can be so easily uncoupled from conversion even in regular meiosis, it is no surprise to find it largely avoided during genetic exchange between repetitive sequences at non-homologous loci, when its occurrence would lead to highly deleterious chromosome rearrangements. But perhaps it is not avoided completely. Spontaneous chromosome aberrations do occur at a low frequency, and something like a "0.1 per cent rule" governing interactions between dispersed repeats could account for many of these.

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SIR — Fink and Petes¹ in writing on gene conversion make repeated reference to a so-called 50 per cent rule. According to this, half the intragenic conversion events in fungi are associated with reciprocal recombination. These authors imply that there is compelling evidence for this conclusion. In reality there is much variation in the frequency with which aberrant segregation is associated with reciprocal exchange, with values ranging from 15 per cent to 75 per cent (see ref. 2, p. 321). Only one example seems to be known of a satisfactory fit with 50 per cent, namely, aberrant 4:4 segregation at the *g* spore

colour locus in *Sordaria fimicola*^{3,4} (and see ref. 2, p. 240).

Klar and Strathern⁵ have discovered that conversion involved in yeast mating type switching during the mitotic cycle is not associated with reciprocal exchange and Klein⁶ has made the same discovery for meiotic intrachromosomal conversion between duplicated inverted sequences at the *HIS3* locus, also in *Saccharomyces cerevisiae*. Fink and Petes¹, in commenting on these results, overlook the evidence of various kinds from organisms as diverse as *Drosophila melanogaster*^{7,8}, *Secale cereale* (rye)⁹ and several fungi¹⁰⁻¹³, that eukaryote recombination may be a two-step process, with gene conversion events in the first step and reciprocal exchange in the second (see ref. 2, pp. 324, 336, 343). The results obtained by Klar and Strathern⁵ and Klein⁶ could then be accounted for if intergenic recombination differs from the normal process in lacking the second step.

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Snookered by colour differences

SIR — Your correspondent William Hickmott (*Nature* **310** 731-732 1984) reported a hitherto unrecognized property of colour. My own part-time socio-physiologico-behavioural research, carried out over many years, lends further weight (*sic*) to the hypothesis proposed, although my extensive data, collected for a different purpose, reveal a different ordering. My research has been on the influence of the recreational context on the liquid consumption of the adult human male.

From the mass (*sic*) of data which I have accumulated I extracted the small subset relating to a particular recreational activity in which pairs of males use sticks to strike small spheres of identical size on a large green rectangular surface. Naturally, in order to minimize the "Hawthorne" effect I have always observed from a great distance (~10³ cm) and have never enquired as to their purpose. (I opine that they are amateur physicists constantly testing and retesting Newton's laws of motion. Their efforts are, however, constantly frustrated since the experimental surface has a number of

roughly circular defects into which spheres frequently disappear.)

All this brings me to the point of my observations. The spheres are of different colours, presumably to facilitate their differentiation in the heavily polluted atmosphere in which these experiments usually take place. The behaviour of these spheres is most curious and for a long time the reason eluded me, but it can be explained now by your correspondent's hypothesis.

The red spheres behave most oddly of all — when they disappear down a defect in the experimental artefact they are not reused, unlike the other coloured spheres. (This correlates well with your correspondent's observations of the red tomato, and suggests that the effect of this colour is so great that the objects are more easily damaged.) As the red spheres disappear the effect of the other coloured spheres is more easily discernible, although the effects are exceedingly complex.

My computer analysis of the results has led me to the inescapable conclusion that the colours do indeed display action at a distance. The disappearance of the colours, usually temporary, triggers a behavioural response in the males which manifests itself in the physical moving of pointers on a scale displayed on the wall near the experiment. I have no idea of the function of these scales, but they may be of symbolic or ritualistic significance.

From my analysis (Imperial undimensional scaling), I conclude that the order of the colours is: red, yellow, green, brown, blue, pink and black, with white occupying an anomalous position on a dimension normal to this. This seems to be almost completely the opposite of the ordering which your correspondent reported, but this need not cause us to reject his basic hypothesis.

The answer clearly lies in the observations discussed by John Maddox (*Nature* **310**, 723; 1984) — the inverse square law needs to be generalized to an inverse sphere law in the complex situation where more than two bodies are involved. As a corollary, the colour of the spheres in the experiments to measure *G* is probably the reason for the variations observed.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a rather technical character which are not provoked by articles or letters previously published (where Matters Arising remains appropriate).

Forest succession

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The once-standard holistic theory of forest succession has more recently been overtaken by a Neodarwinian reductionist theory with defects of its own. A less inflexible theory is much needed.

THE term succession is used to describe many types of vegetation change on widely different scales in both space and time. In discussing forested or potentially forested zones, succession is formally defined here as the directional change with time of the species composition and vegetation physiognomy of a single site where climate remains effectively constant. Understanding succession is important for two reasons—the value of the concept in the development of ecology as a science and its enormous potential in the development of programmes for the conservation and exploitation of biological resources^{1,2}. Succession is nevertheless a most controversial issue, involving confrontation between holism (the view emphasizing unity and integration in nature), and reductionism (in which chance and darwinian interpretations dominate).

Primary successions occur on previously unvegetated terrain. Glacial moraines in Alaska, for example, eventually support spruce forest after first being colonized by herbaceous plants, which are succeeded by thickets of willow and alder. Secondary successions occur on disturbed areas, including those converted to agriculture and subsequently abandoned. In parts of the east coast of the United States, the herbaceous communities of such 'old fields' are quickly invaded by pines and then by oak, hickory and other species characteristic of the original forests of the region. Environmental changes occurring during succession are classed as autogenic when they are a consequence of the presence of plants. Examples are the accumulation of organic matter in soil and the modification of light levels within vegetation. Allogenic changes, on the other hand, are those caused by factors external to the vegetation. Examples include the deposition of silt by rivers and local temperature changes as glaciers retreat.

No direct study of forest succession has so far been possible because of the longevity of the organisms involved. Descriptions of succession are usually inferred from studies of the vegetation of adjacent sites of different known ages, in which case it is necessary to assume that sites differ only in age and not in substrate material, climatic history, past disturbance or the input of propagules. Although often reasonable, the validity of this assumption is not always carefully assessed³; descriptive field studies rarely provide much insight into the mechanisms of successional change, which remain hypothetical and are a major concern of the long-standing theoretical controversy over succession.

Theories of succession

The development and interrelationships of holistic and reductionist theories have been reviewed by McIntosh^{4,5} and these are briefly summarized and contrasted here. Common to both theories, however, is the natural history of trees. The broadest distinction that can be made between tree species participating in successions is between 'pioneer' trees, those species usually most prominent on open sites and absent from forest, and 'forest' trees, individuals of which contribute to the canopy of mature

forest. The classification^{6,7}, summarized in Table 1, may be a 'crude oversimplification' (see ref. 8), but it is necessary for presentation of the general arguments on succession⁹.

Holistic ecology takes the ecosystem, a unit of environment and biota in reciprocal interaction¹⁰, as its object of study. Ecosystems are held to possess 'emergent' properties—properties which cannot be inferred from those of the parts of the system. Successional change is often considered to occur in stages through wave-like invasions by groups of species, and is thought to be controlled by the vegetation itself. Thus autogenic change, caused by the presence of a particular species or group of species, makes the environment suitable for the next group of species and so facilitates successional change. This is the 'facilitation' hypothesis. In holistic theory, the development during succession of 'biological control' of nutrient cycling is also emphasized. Succession is interpreted teleologically as the process of development of an ecosystem of maximum stability (by the yardstick of resistance to disturbance) and of maximum efficiency in the utilization of resources¹⁰. Successional change is usually held to be orderly, predictable and therefore deterministic, converging to forest from various different starting points.

Table 1 Empirically determined attributes of pioneer and mature forest tree species

Characteristic	Pioneer tree	Forest tree
Dispersibility of seed	To long distances wind/bird/bat	Short distance rodent/bird/none
Seed weight	Light to heavy	Relatively heavy
Seed germination: light-stimulated	Yes	No
inhibited by far red light	Yes	No
Longevity of individual	Shorter	Longer
Time to repro- ductive maturity	Shorter	Longer
Height growth	Fast	Slow
Height at maturity	Shorter	Taller
Resource acqui- sition rates	Fast	Slow
Photosynthesis light-saturated at	High light intensities	Low light intensities
Recovery from resource limitation	Fast	Slow

These categories represent the extremes of a spectrum of ecologies; pioneer is analogous to 'r-selected', forest to 'k-selected'. Reductionist authors assert that these differences between pioneer and mature forest species explain all the phenomena of succession (see also Fig. 1) (from refs 7 and 8).

In the past decade, the defects and lack of applicability of holistic theory have been convincingly exposed and a new body of reductionist theory has developed¹¹⁻¹⁶ based on Gleason's interpretation of the plant community as a fortuitous assemblage of species populations, each with a unique behaviour¹⁷. The fundamental stochasticism of this view is supported by evidence of several alternative successional pathways within a given vegetation type¹⁸. The cornerstone of the new successional theory is the modern interpretation of Egler's¹⁹ initial floristic composition (IFC) model (Fig. 1). In this model all the species participating in a succession become established at or very shortly after initiation. Succession is merely the sequential physiognomic dominance of the site by species with different life histories, growth rates and sizes at maturity. The facilitation hypothesis is rejected, and autogenic changes are seen as neutral or inhibitory factors rather than the driving force of succession. The theory emphasizes that the availability of resources may decrease with succession as the density of individuals increases. Pioneers are seen as opportunists exploiting environments free from 'competition', while forest trees are efficient and conservative in their use of resources. This interpretation of autogenesis has resolved into two subsidiary models, 'tolerance' and 'inhibition', considered more fully below. Although a reductionist, darwinian approach^{20,21} has given great impetus to contemporary plant ecology, enjoying the heuristic advantages inherent to reductionism, the new successional theory has developed independently of this mainstream and rests not on new empirical findings, but on reinterpretations of existing data. Unease has already been expressed at this development^{4,5,22}; is there progress if models such as IFC, tolerance and inhibition simply replace the holistic framework as the dogma by which inadequate field data are interpreted?

This review is a critical appraisal of the theory as it now stands, and suggests a better approach in considering succession. The ideas developed in references 11-18 will be referred to as the 'reductionistic approach' and their authors as 'the reductionists'.

Reductionist succession

Tolerance is the ability of individuals (particularly of juveniles) to survive in the poor environmental conditions resulting from the high density of other individuals. Foresters have long been aware that tree species differ in their ability to become established and grow within a forest^{23,24}. Although this may be due in part to factors affecting the availability of soil moisture and mineral nutrients, tolerance has come to be synonymous, in successional theory, with the ability to persist or grow in shade. By this definition, pioneer species are intolerant and forest species tolerant, because pioneer trees cast shade, thereby preventing their own regeneration and are replaced by forest trees, which can become established in the shade. It is well known that felling stands of intolerant pines on old fields increases the growth of the tolerant juvenile forest trees in the understorey, raising the suggestion that autogenic change (shading) delays rather than facilitates succession¹¹. Unfortunately, however, removing the understorey also increases the growth of pines²⁵, introducing the first constraint on this simple model.

Some have nevertheless unacceptably adopted tolerance as the sole mechanism of forest succession^{26,27}. Tolerance is clearly an essential component of the biology of forest trees. However, it being the only mechanism of succession depends on whether environmental factors other than quantum flux are limiting to establishment and growth, whether the states of these factors change as succession proceeds and whether pioneer and forest species differ in their responses to these environmental changes. At the outset, the possibility must at least be considered that juveniles of some forest trees not only tolerate the environment of a pioneer forest canopy, but require it. Silvicultural experiments in the rain forests of Malaysia, for example, have shown a wide variation between species in the responses of apparently suppressed saplings to the clearance of canopy trees⁸. The growth of some species accelerates, as the tolerance model

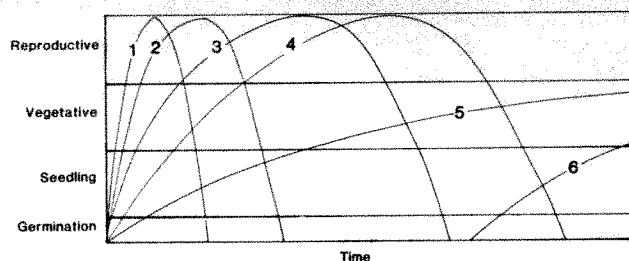


Fig. 1 Schematic representation of the initial floristic composition model of succession. Species 1-5 become established at the initiation of the succession, and successively dominate the site due to the different lengths of time they take to reach maturity. Species 1 may represent an annual herb, species 4 a short-lived shrub, and species 5 a slow-growing, shade-tolerant forest tree. Species 1-4 fail to regenerate as they are light-demanding. Late colonization by species 6 occurs as a consequence of the release of the inhibitory effects upon it of species 3 (modified from Gómez-Pompa and Vásquez-Yanes³⁹).

predicts, but individuals of other species become stunted, susceptible to disease or predation, or are killed.

The inhibition model proposes that the vegetation occupying a site prevents the invasion of other species. The best evidence for this model is the demonstration of apparent long-term stability in certain vegetation types, mainly monospecific stands of clonally spreading shrubs¹³. These areas may not be typical; they are usually small or extremely small, and sometimes have an unusual management history. Thus inhibition may not be a general phenomenon in forest succession; more work is required to establish the frequency with which it occurs.

Initial floristic composition

Initial floristic composition (IFC) has become accepted as a descriptive model for both primary^{4,16} and secondary successions¹¹. Although this model fits field observations more closely than did holistic ideas, it is certainly not an explanation, nor even a general description, of succession. Niering and Goodwin suggest¹³ that, with respect to North American old field successions, IFC requires 'conceptual elaboration' and go on to include the supposedly more general cases in which woody species are not present at the initiation of successions, but appear within a few years. This elaboration neither describes nor explains the behaviour of many forest tree species.

There is a substantial body of information about old field successions in several forest regions of the United States. Two groups of woody species consistently appear within 10 years of the abandonment of a field. The first comprises short-lived shrubs and small trees, and is exemplified by the species of *Prunus* and *Juniperus* recorded on recently abandoned fields in New Jersey²⁸⁻³⁰. The second group consists of larger and longer-lived trees which may also occur as components of mature forest. In the Carolinas and southern Virginia, this group includes loblolly pine (*Pinus taeda*) and shortleaf pine (*Pinus echinata*), which may appear in large numbers after one year, the density of seedlings depending on the proximity of parent trees^{31,32}. Red maple (*Acer rubrum*) behaves similarly²⁹. Despite the claims of Niering and Goodwin¹³ and others, it is clear that almost all the oaks (*Quercus* spp.) and hickories (*Carya* spp.) characteristic of the mature forests of many of the central and eastern states of the United States are significantly later colonizers of old fields. Colonization by oaks has been recorded between 11 and 20 years after the abandonment of fields²⁸⁻³⁶, except in two cases in which very early colonization was induced by the presence of overhanging parent trees^{37,38}. Longer periods of time elapse before the appearance of hickories; sites abandoned for 40 and 60 years are amongst the most recent from which new individuals have been reported^{33,34}. (The one exception is a record of hickory

seedlings after three years on one of the 10 sites monitored over 11 years by Buell *et al.*²⁸, but further colonization has not been reported during this continuing study³⁰.) In all the cases cited, colonization by oaks and hickories occurred on sites already supporting established pioneer vegetation.

Studies of secondary forest succession in the humid tropics have been ignored by the reductionists. The 'old fields' of the tropics are forest clearings abandoned by farmers practising shifting cultivation. It is generally accepted that species of late successional and mature forests do not colonize such clearings until there is already a well established pioneer vegetation, dominated by fast growing pioneer trees^{2,8,9,39-41}.

In England, primary successions in abandoned quarries recently received some attention in a study of the vegetation of three chalk quarries⁴². Birch (*Betula pendula*) and willows (*Salix* spp.) are able to behave as pioneers on bare chalk, while hawthorn (*Crataegus monogyna*) and English oak (*Quercus robur*) are clearly later colonizers, appearing once the sites already support pioneer scrub (Fig. 2). A survey of 48 chalk and limestone quarries throughout the country suggests that this pattern of colonization has been repeated several times⁴³.

On many sites undergoing succession, the seedlings of shrub and tree species such as *Crataegus* and *Prunus* spp., and the tropical genus *Cecropia*, exhibit markedly clumped distributions, occurring mostly under the canopies of established trees^{42,44-49} (see Fig. 3). These species may be very early colonizers of abandoned sites, such as orchards which already support trees^{44,45}, or later colonizers in primary successions, such as those occurring in abandoned chalk quarries⁴².

Different tree species seem not to have become established simultaneously at the initiation of any of the successions described here, so that the simulational and predictive power of IFC would appear to be limited. Differential seed dispersal is probably chiefly responsible for the failure of this model, but this does not imply that it is sufficient to regard dispersal simply as a stochastic process in which light wind-dispersed seeds will usually go further in a shorter time. The role of animal dispersal vectors will be crucial to the colonization of successional sites by many woody species. Of the pioneer trees mentioned, the pines, maple, birch and willow all have wind-dispersed seeds⁵⁰, while those of tropical forest pioneer trees are widely dispersed by birds and bats^{51,52}. Oaks, hickories, other temperate forest species such as beeches, and many of the trees of undisturbed humid tropical forests have large short-lived seeds^{50,53,54} which lack the fleshy pulp and other characteristics of seeds specifically attractive to dispersal vectors, but which are nevertheless dispersed by the hoarding behaviour of forest rodents and birds⁵⁵. It is probable that many such forest vertebrates do not enter open fields or clearings except on brief forays from surrounding hedgerows or forest, as in the case of the red squirrel (*Tamiasciurus hudsonicus loquax*)⁵⁶. Acorns are a major component of the diet of the jay (*Garrulus glandarius*), which buries them in forest, open woodland or around isolated trees, but avoids open fields^{57,58}. The genera *Crataegus*, *Prunus* and *Cecropia* all bear fleshy fruits, dispersed by frugivorous birds (and bats, in the case of the tropical taxon). It is likely that in all these cases, colonization of a site requires the presence of trees in which the dispersal vectors may roost^{42,44-49}; similar considerations may apply for the dispersal of the many species of canopy trees in humid tropical forest.

These observations are consistent with the hypothesis that the presence of pioneer woody species often increases the rate of colonization of a site by other woody species whose seeds are dispersed by animals and birds since the pioneers render the site more attractive to dispersal vectors (see also ref. 59). The behaviour of the vectors is probably decisive; bird-dispersed shrubs do themselves behave as pioneers on old fields in the United States^{28-30,60} and in England⁶¹. The testing of this hypothesis would be welcome.

The few known successions which actually resemble IFC are of two types. The first is where forest species with wind- or ballistically-dispersed seeds dominate the local flora, as in the

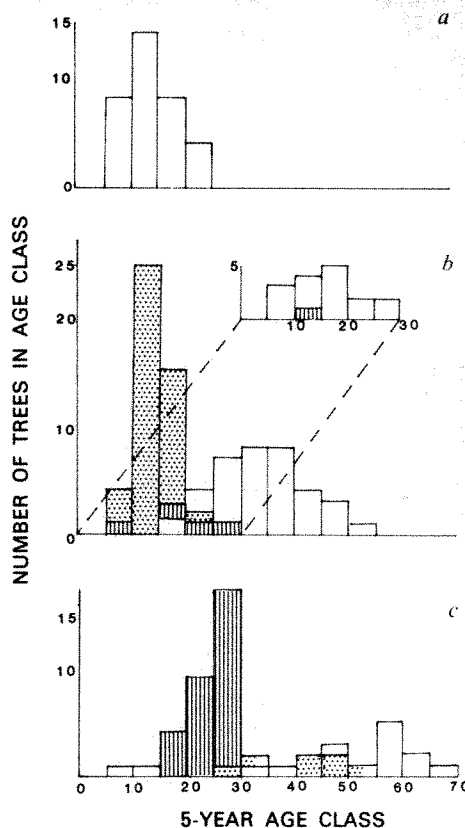


Fig. 2 Succession in abandoned chalk quarries in southeastern England. Histograms represent the age structures (excluding first year seedlings) of populations of birch (*Betula pendula*, open bars), oak (*Quercus robur*, vertical lines) and hawthorn (*Crataegus monogyna*, stippled) in representative stands on the three adjacent quarries, in August 1982. Vegetation was homogenous at the scale sampled, removing species/area effects. a, Site abandoned for approximately 7 years; b, site abandoned for ~35 years; c, site abandoned for ~60 years. Birch is a primary colonizer, appearing before abandonment of the sites. Oak and hawthorn are much later colonizers; oaks in c were well formed, some attaining the height of the oldest birches. Seedlings of hawthorn were recorded in a in 1982 (Fig. 3). The inset in b records another part of this site and illustrates spatial variation in colonization. Although these data show populations each with an individualistic behaviour, they do not conform to the IFC model.

upland evergreen forests of Ghana⁶², on mudflows in Californian river valleys⁶³, and on Alaskan glacial moraines. (Other factors affecting moraine successions, however, would seem to limit the value of IFC in even this case, as will be shown below.) The second is the regrowth of forest after clear-cutting, when the succession is dominated by pioneer trees from viable seeds already present in the soil and the regrowth of forest species from stumps⁶.

The role of autogenic change

Implicit in the reductionistic approach is the assumption that different tree species are equally able to become established and develop normally in the environment of a newly available site. The reductionists, however, carefully do not analyse contradictory evidence, probably because it leads towards the facilitation model. Drury and Nisbet cite undeniable evidence for the role of allogenic change¹¹, but their attempt to refute the hypothesis that autogenic soil development plays a role in primary succession does not succeed. Botkin emphatically concludes that the elements of the reductionistic framework (as defined here) are sufficient to account for all the phenomena of succession⁶⁴. His computer simulations reproduce the growth of established individuals of different tree species on mature soils, so that it

is scarcely surprising that environmental changes other than shading were found to be unimportant. A large body of observations made in other circumstances remains to be incorporated into the reductionistic framework. Part of the difficulty is that the evidence is scattered in a wide range of sources, and is not always directly related to succession. This is unavoidable in a field where progress towards understanding the mechanisms at work has only just begun⁶⁵.

Harper provides a basis for understanding the recruitment of seedlings²¹. A germinating seed occupies a 'safe site', a microenvironment which provides, for example, dormancy-breaking stimuli, the resources necessary for germination (particularly water and oxygen) and freedom from predators and pathogens. Microenvironmental heterogeneity can have dramatic effects on seed germinations which varies between species in a manner related to the different sizes and shapes of the seeds. Environmental factors affecting the survival of seedlings include the availability of moisture, temperature variations and predators. All of these factors may be modified by the presence of neighbouring plants, and each is subject to autogenic change during succession. The accumulation of organic matter in the surface layers of the soil during forest succession on old fields and the floors of abandoned chalk quarries increases the retention of moisture in the zones most affecting seeds and seedlings^{32,42,66}; this is probably a feature of all successions in which organic soil horizons develop. In the humid tropics, diurnal soil and air temperatures and saturation deficits of the air, are all lower in forests than in open clearings, largely because of shading⁶⁷⁻⁶⁹. Indeed, such spatial environmental heterogeneity is a general feature of forest microclimates⁷⁰, and the kind of environmental changes to be expected during succession can be extrapolated from these findings. Pioneer and forest species may, however, differ in their responses to these autogenic changes, as will now be examined.

Generalizations are not possible from studies of the environmental requirements and tolerances of pioneer trees colonizing clear-felled forest sites where removal of forest trees is assumed to increase the availability of water and mineral nutrients. The pioneer trees of primary successions do not enter an environment with the freely available resources of clear-felled forests; neither, it would seem in some cases, do those colonizing old fields.

Within young, primary successional birch woodland (Fig. 2, site b), the mortality of seedlings of birch and willow is higher on bare chalk than in gaps in a turf of mosses and herbaceous plants⁴². In a neotropical secondary succession, the recruitment on bare soil of seedlings of the pioneer tree *Cecropia filicifolia* was markedly increased by treatments which reduced surface temperatures and evaporation⁷¹. It seems certain that tolerance of periodic drought and other environmental extremes is a critical factor in the survival on old fields of pioneer loblolly pine, red cedar (*Juniperus virginiana*) and elm (*Ulmus alata*)^{72,73}. (An important component of the biology of seedlings of pioneer species is that they should be tolerant of, or have a mechanism to avoid, high leaf temperatures induced by short-wave solar radiation at exposed sites⁷⁴.) Examples of sites which become dominated by pioneer species suggest that this occurs by toleration, not by exploitation of the environment.

There are very few critical studies on the establishment of forest trees in pioneer environments but there is much evidence to suggest that forest species do not have the ability of pioneers to become established there. Acorns are readily killed by heat, desiccation or winter frost when surface-sown on open sites⁷⁵⁻⁷⁷. Germination requires burial, to protect acorns against environmental extremes, to facilitate uptake of water and to protect them from seed predators, a major cause of mortality⁷⁶. These characteristics are probably shared by all large tree seeds, providing the first potential barrier to establishment on open fields and clearings in temperate and tropical zones.

The next potential barrier may occur at the seedling stage since seedlings of red and white oaks (*Quercus rubra* and *Q. alba*) are intolerant of drought and do not survive on drier forest sites⁷⁸; survival of red oak seedlings planted on coal spoil

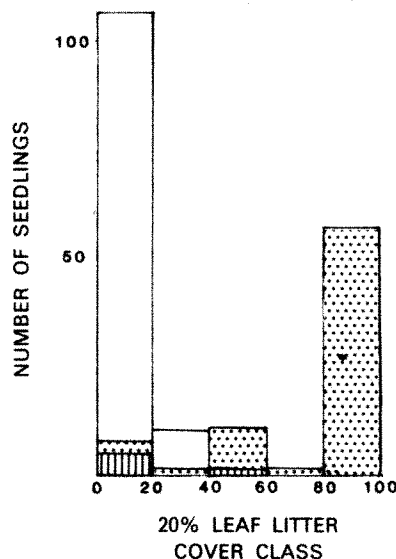


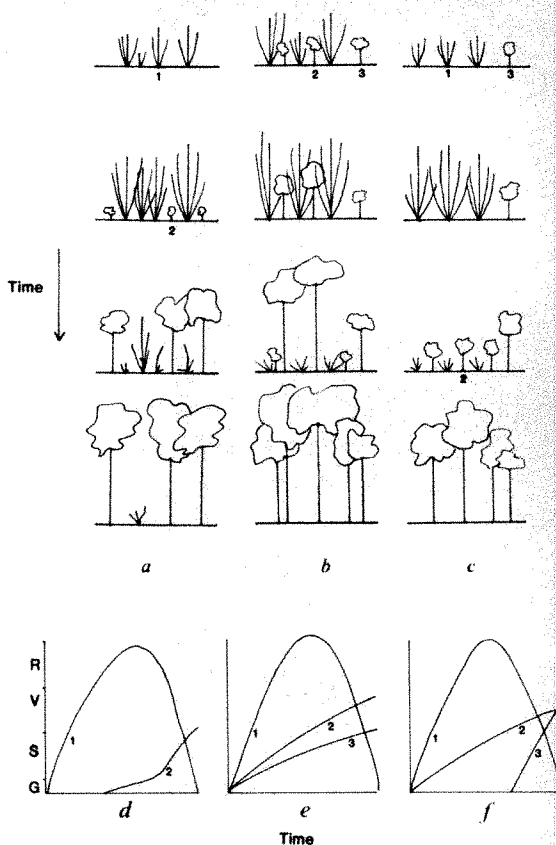
Fig. 3 Separation of seedlings of three tree species along a spatial gradient in ground cover of leaf litter in site a of Fig. 2, September 1982. Data are summarized from 60 quadrats of 0.25 m², 10 randomly positioned in each of six 25-m² stands of birth/willow scrub. All quadrats in the 81-100% litter cover class were directly under the canopies of willow bushes. The seeds of birch (vertical lines) and willow (open bars) are widely dispersed by wind, but bare ground is required for the establishment of seedlings of these species. The distribution of hawthorn seedlings (stippled bars) is probably a consequence of patterns of seed deposition by frugivorous birds perching in established bushes.

is greatly improved by treatments reducing the high surface temperatures and the incidence of moisture deficits⁷⁴. On a Michigan old field, seedlings of *Quercus velutina* and *Q. alba* survived when established among herbaceous vegetation, but not on bare ground⁷⁹. Even within forest, seedlings of beech (*Fagus grandifolia*) occur much less frequently on dry south- and west-facing slopes than on those facing north and east⁸⁰. European beech (*F. sylvatica*) does not establish from seed on open ground⁸¹; *Agathis australis* in New Zealand requires the presence of associated shrubs⁸². Shade may be required for the successful establishment of seedlings of tropical forest trees of the family Dipterocarpaceae⁸, possibly as a consequence of intolerance of drought and high saturation deficits, and of the high leaf temperatures likely to be experienced in full sunlight.

Traditional theories assign autogenic development of soil and mineral nutrient cycling a functional role in two types of forest succession—secondary successions on abandoned shifting cultivation clearings in humid tropical forests, and primary successions on substrates such as glacial moraine. In the former type, forest clearings are abandoned when they will no longer produce a useful crop, partly because of loss of soil fertility. The ecological implications of this have been discussed². After abandonment, the site is often invaded by pioneer trees and improvements in soil fertility take place, which has been demonstrated on sites in Nigeria and Ghana^{83,84}. Sites where such an increase in soil fertility does not occur, such as are found in Costa Rica⁸⁵ where the soils are unusually fertile⁸⁶, may well be atypical. Extrapolating from Jordan and Herrera's⁸⁶ conclusions, it is possible that in the humid tropics facilitation may operate in successions on less fertile soils such as those of the Amazon basin, which are intensely leached. Much work remains to be done to resolve this issue.

The major early constraints on plant growth during primary succession are common to all environments; a lack of available macronutrients such as nitrogen and phosphorus and a lack of soil organic matter^{42,59,87-98}. In physically extreme environ-

Fig. 4 Composite mechanisms of succession. The tolerance model operates in all examples, as light-demanding pioneer trees fail to regenerate. Inhibition, tolerance and facilitation affect the colonization and development of forest trees. *a*, Seed dispersal vectors of a large seeded forest tree do not enter a site until pioneer vegetation has developed sufficiently to provide a habitat attractive to them. The seeds germinate but the seedlings do not develop in the environment of the pioneer vegetation; the seedlings are, however, protected from the potentially deleterious effects of direct sunlight. The seedlings develop to maturity on the deaths of individual pioneers. Facilitation operates at the seed dispersal stage, tolerance and facilitation simultaneously at the seedling stage. *b*, A nitrogen-fixing pioneer and a forest species become established simultaneously on an extremely infertile mineral substrate. Shade-tolerant juvenile forest trees in the pioneer thicket benefit from the nitrogen fixation and other environmental changes consequent on the presence of the pioneer. Open growth forest trees develop more slowly, and are overtopped both by 'nursed' individuals and by later colonizers. Facilitation and tolerance operate at the juvenile stage, although open grown forest trees may also develop to maturity. *c*, A similar situation to *b*, but forest trees are unable to become established in the pioneer thicket, colonizing the nitrogen-rich environment left after the deaths of the pioneers. Inhibition operates at the establishment stage, facilitation at the juvenile stage. *d-f*, The population patterns associated with each model. Although these are similar to IFC (Fig. 1), the actual ecological process that the diagrams describe is the outcome of more than simply differences in the life-history characteristics and shade tolerances of the species concerned. 1, Pioneer tree; 2, nursed or late colonizing forest tree; 3, open grown forest tree; vertical axis as in Fig. 1.



ments, nitrogen-fixing herbs, shrubs (for example, *Dryas drummondii*) and small trees (for example, the alders, *Alnus* spp.) occur as pioneers or very early colonizers in successions on basalts in Japan⁸⁷ and glacial moraines in Switzerland, Alaska and Canada⁸⁸⁻⁹⁸. These species must be largely responsible for the marked increases in the amounts of nitrogen and organic matter in the substrates of moraines of increasing age, the factor traditionally assumed to facilitate other species. One exception is the Yukon Territory⁹⁶⁻⁹⁸ where the short growing season is probably the dominant ecological factor.

When it was realized that floristic descriptions of primary successions often resemble IFC^{4,11}, facilitation was then held to have been rejected as an explanation of succession. The crucial point, however, is that the different models of succession are not mutually exclusive. Even where seedlings of forest trees occur in a pioneer environment, both the probability that they will survive and the rate at which they develop to maturity may be increased by continuing autogenic changes. Cooper, for example, is convinced that pioneer spruce (*Picea sitchensis*) at Glacier Bay, Alaska, were permanently stunted and overtopped by later colonizers encountering less extreme conditions⁸⁹⁻⁹¹. Other workers have described symptoms of poor growth, chronic nitrogen deficiency and the probability of high mortality in seedling and juvenile cottonwood (*Populus trichocarpa*), spruce and hemlock (*Tsuga mertensiana* and *Tsuga heterophylla*) on recent moraines⁹²⁻⁹⁴. These observations, however, do not point unambiguously to either holistic model for primary successions, and await explanation. Experimental studies on natural vegetation are clearly required; attention has, however, been drawn by Bradshaw to the possible functional similarities between the environmental changes of primary successions and those brought about artificially through land reclamation work⁹⁹ (although his generalizations about the amounts of nitrogen required in the 'ecosystem' before it will support trees remain to be substantiated). Nitrogen-fixing shrubs and trees are widely used as nurse crops for less robust species while the soil-improving properties of alders have long been appreciated¹⁰⁰. Thus *Alnus sinuata* is recommended as a nurse crop for douglas

fir (*Pseudotsuga menziesii*) on sites low in nitrogen and organic matter¹⁰¹. In New Zealand, a nurse crop of tree lupin (*Lupinus arboreus*) is essential to the establishment of *Pinus radiata* on sand dunes¹⁰².

In conclusion, the generalization that because of the absence of 'competition', early successional environments offer greater levels of resources than do forests, and the corollary that pioneer trees are exploitative and forest species conservative and tolerant of resource shortages, is not supported by the evidence. Often pioneer trees show tolerance, not of shading, but of the extreme temperatures and infertile, often drought-prone substrates of many early successional environments. Forest trees may be tolerant of 'competition', but may well not be tolerant of physically hostile sites. Indeed, there is no reason to suppose that the two types of tolerance are conferred by the same biological attributes. Pioneer trees cause environmental changes which may alleviate constraints on the establishment of forest trees in initially adverse environments. But such a hypothesis and the elements of the reductionistic framework are not mutually exclusive.

The third alternative

The need for a darwinian approach to the analysis of forest succession is beyond dispute. Neither reductionist nor holistic theories of succession have produced models which explain field observations. Yet because the reductionist framework has been described in terms of the Neo-darwinian evolutionary synthesis, it tends to be uncritically accepted. As Gould¹⁰³ has pointed out, the urge to tell (and to believe) a good story affects not only holists.

In essence, the reductionists hold that succession occurs because of the existence of a spectrum of types of life history, which is the basis of the IFC model. Autogenic change is allowed a subsidiary role when its effects are seen as 'negative', as in tolerance and inhibition models. Much importance is also assigned to stochasticism, a disorganizing power as elusive as the emergent properties of the holists¹⁰⁴. The theme of this article has been that the reductionistic story fails, as it stands, for two

reasons: it neglects the nature of seed dispersal, and its generalizations about environmental change and exploitative and conservative species are untenable.

A third, synthetical approach should be sought, if only as a stimulus to further investigations. Life histories and tolerance will be important factors in any model of forest succession, but they cannot be assumed to provide a complete explanation of the phenomenon. In considering which other mechanisms are involved, it is not necessary to view facilitation as inseparable from the other elements of holism, particularly its teleological character. It is in fact adequate to suppose that the fitness of a pioneer tree is dependent only on its ability to colonize, grow and produce seed in early successional environments. The consequent environmental changes and the effect on other species are secondary results of this process. The facilitation model should allow that certain autogenic environmental changes may increase the input of seeds, the establishment of seedlings or the growth and survival of established individuals of forest species. Facilitation, tolerance, inhibition and allogeneses are interdependent mechanisms in succession and may affect the same individual successively or simultaneously during its life cycle (Fig. 4; refs 89–91). The relative importance of the various

mechanisms is likely to vary widely between environments, but attempts to classify succession on the basis of a single underlying mechanism only obscure understanding and inhibit progress.

Reductionism has not solved the succession problem, and will not do so while it relies on the same deduction from inadequate general models which led to the fall of the holistic scheme. For progress, the induction of general theory from fieldwork based in the observational and experimental techniques of plant population biology^{20,21} would seem to have much to recommend it (see ref. 105; unfortunately, not a study concerned with succession). This recalls the problem of time-scale, although many of the problems outlined above could be solved by relatively short-term studies. However, such an approach has the great advantage of theoretical agnosticism and is the least value-laden available. Any other way forward is difficult to see; the general models that have been freely proposed so far are wholly inappropriate in relation to the quality and quantity of available data.

I thank P. J. Grubb, T. A. Rowell, H. J. Harvey, P. Greig-Smith, L. Guarino and J. Mitchley for their interest and time. This work forms part of a study funded by a NERC/CASE research studentship with Thurrock Borough Council.

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A model for the evolution of the Grampian tract in the early Caledonides and Appalachians

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Analogy with models developed for Newfoundland suggests that the ophiolites of the Grampian tract were emplaced as hot, young oceanic crust. The conversion of a mid-ocean ridge-fracture zone system into a subduction zone resulted in the northward emplacement of a giant ophiolite nappe onto the continental margin in the early Ordovician. This obduction occurred long before the collision between the Laurentian and Baltic Shields. The general features of this model may be characteristic of orogens resulting from oceanic closure.

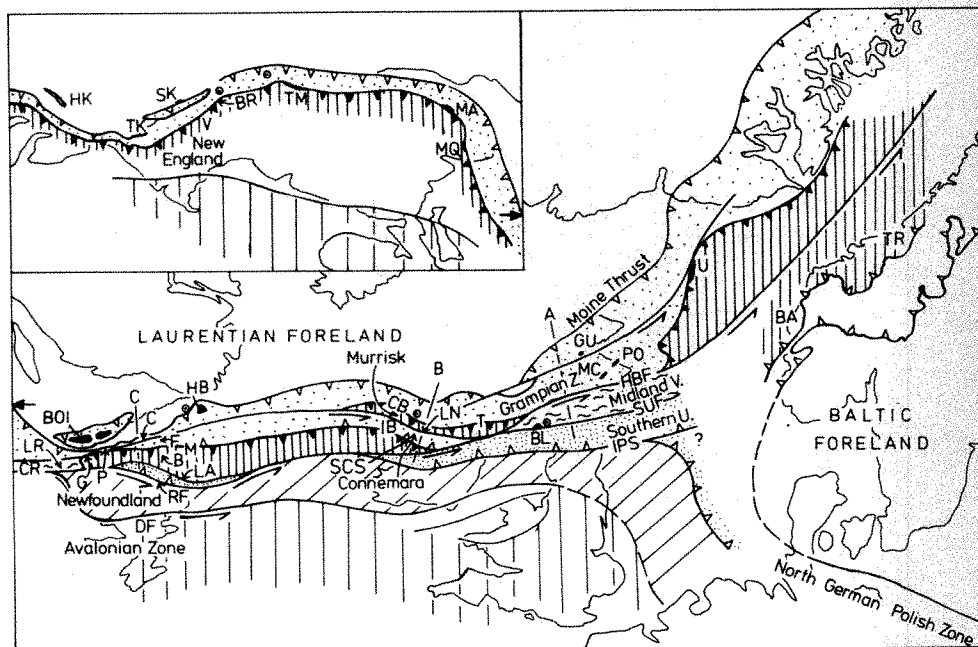
OROGENIC contraction occurs in at least seven ways: (1) above subduction zones at continental margins (Andes); (2) on compressional bends of transform faults (Transverse Ranges); (3) by the compressional collapse of intracontinental extensional zones (Alps); (4) by continent-continent collision (Himalayas); (5) by arc-continent collision (Taiwan); (6) by the collision and lateral displacement of oceanic plateaus and other exotic terranes (North American Cordillera); and (7) by ophiolite obduction (Oman). The earliest interior deformations usually involved ophiolites and blueschist metamorphism, without synchronous flysch or molasse deposition in exterior zones and, therefore, probably occurred below sea level. Collapsed extensional zones have crystalline basement rocks above attenuated subcontinental mantle in the highest nappes, and lack well developed ophiolites. In orogens resulting from the closure of oceans, the highest nappes are ophiolitic, obduction preceding terminal collision

as a distinct event affecting margins. This mechanism was responsible for the evolution of the early Caledonides and is developed here.

The Grampian Orogen

There is now general agreement that the Northern Appalachian/Caledonian orogen evolved by the closing of the Iapetus Ocean¹, with terminal collision in the late Silurian/early Devonian in the Caledonides and in the mid-Devonian in the Northern Appalachians²⁻⁴. The two sides of the orogen have quite different basements, rifting and subduction histories and their only common feature was their 'stitching together' across the Iapetus Suture. The plate tectonic setting of the Grampian tract and its Appalachian equivalents (Fig. 1) has been extensively debated and hypotheses have fallen into three classes: those involving an Andean-type setting above a north-west-

Fig. 1 Simplified tectonic map of the Northern Appalachians and British Caledonides restored to the pre-Atlantic continental configuration of Bullard *et al.*⁵². Inset map shows the southwestward continuation of the Appalachians into New England. B, Betts Cove Ophiolite Complex; BA, Bergen arcs; BL, Ballantrae Ophiolite Complex; BOI, Bay of Islands Ophiolite Complex; BR, Brompton Line; C, Coney Head Complex; CB, Clew Bay Ophiolite Zone; CR, Cape Ray ophiolites; DF, Dover Fault; F, Fleur-de-Lys; G, Glover Island ophiolites; GU, Glen Urquhart; HB, Hare Bay Ophiolite Complex; HBF, Highland Boundary Fault; HK, Hamburg Klippe; IB, Inishbofin; IPS, Iapetus Suture; LA, Lukes Arm Fault; LN, Lough Nafoeey Fault Zone; LR, Long Range; M, Mings Bight Ophiolite Complex; MA, Mount Albert; MC, Morven/Cabrach; MQ, Macquarau; P, Pasadena; PO, Portsoy; RF, Reach Fault; SCS, South Connemara Series; SK, Starks Knob; SUF, Southern Uplands Fault; T, Tyrone; TK, Taconic Kippe; TR, Trondheim; U, Unst Ophiolite Complex; V, Vermont ultramafic belt. [] , Grenville basement within the orogen; [] , Infracambrian to early Ordovician sequences in Grampian zone; [] , Ordovician arc terranes; [] , Ordovician/Silurian accretionary prisms; [] , main ophiolite suture contact bounding Ordovician arc terranes; [] , major thrusts; [] , ophiolite complexes; [] , oceanic and arc terranes south of the Iapetus Suture; [] , Avalon/Cadomian terrane. Continuity of zones and terranes between Newfoundland and Ireland implies only tectonic equivalence and not necessarily physical continuity.



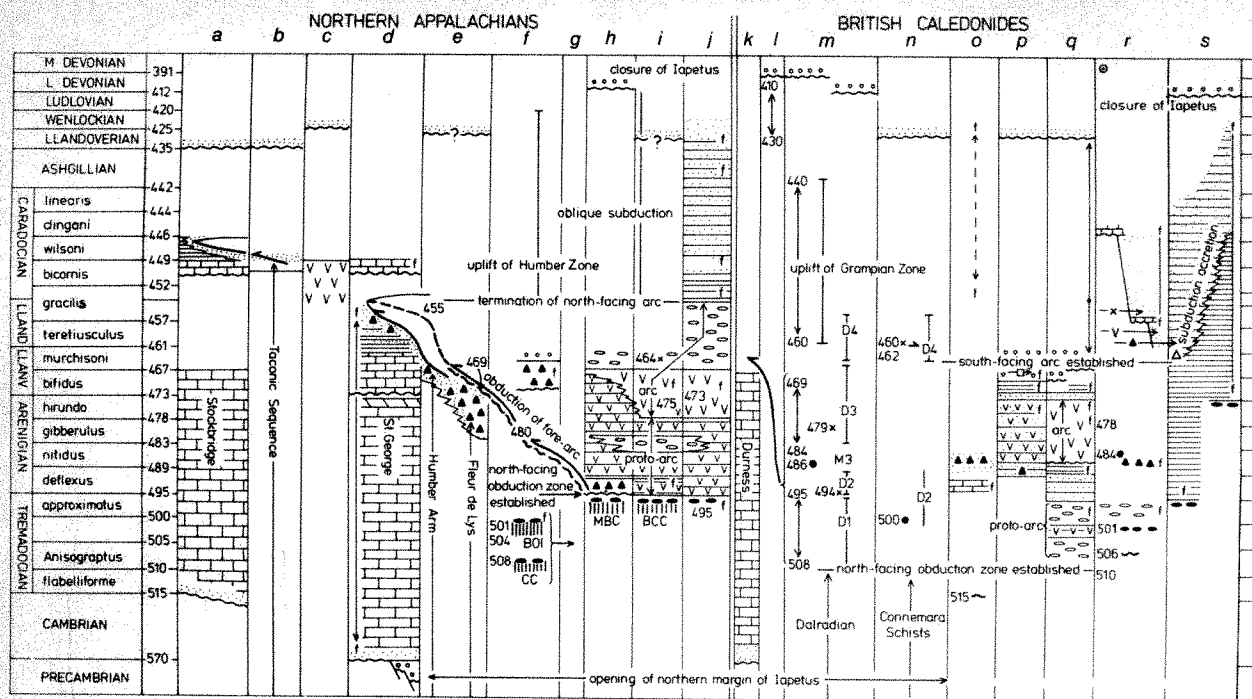


Fig. 2 Stratigraphical summary of facies, structural, igneous and metamorphic events, and isotopic ages in the orotectonic zone of the Northern Appalachians and British Caledonides (north to the left). *a-c*, Eastern New York/Vermont; *d*, West Newfoundland Platform; *e*, Humber Allochthon and western Fleur-de-Lys sequence; *f, g*, Bay of Islands and Coastal Ophiolite Complexes and Baie Verte Zone; *h-j*, eastern Burlington Peninsula and Notre Dame Bay; *k*, north-west platform; *l*, Moine; *m*, Dalradian; *n*, Connemara; *o*, Highland Boundary Fault Zone; *p, q*, South Mayo Trough; *r*, Girvan/Ballantrae; *s*, Southern Uplands. [Limestone], [dolostone], [shale/mudstone], [marine clastics], [turbidites], [non-marine clastics], [volcaniclastics], [intermediate/silicic volcanics], [mafic/intermediate volcanics], [ophiolite detritus], [seamount accretion], [ophiolite pillow lavas], [sheeted dyke complex/gabbro/ultramafics], [thin wavy line, unconformity], [thick wavy line, ophiolite basal emplacement aureole], [f, fossil control], [x, mafic intrusions], [x, intermediate/silicic plutons], [□, metamorphic detritus], [○, block moving away along terrane boundary], [●, block moving towards along terrane boundary]. Isotopic ages for *a-j*: 455, basal aureole⁵³; 464+5, Burlington Granodiorite; 469+5, basal aureole⁵⁴; 470, Causeway Diorite⁵⁵; 473, Twillingate dykes⁵⁶; 475+10, Cape Brule Porphyry⁵⁷; 480, basal aureole⁵⁸; 494+5, Brighton Gabbro⁵⁹; 501+13 and 508+6, Bay of Islands Complex⁶¹; 504+10, Bay of Islands Complex⁶¹; 508+5, Coastal Complex⁶². *l, m*: 410-430, Moine cooling ages; 440-460, Dalradian cooling ages; 469-484, Itlay Nappe cooling ages; 479+12, Ardlethen granite; 495-508, low-grade southeastern Dalradian²³; 486+17, Newer Gabbros⁶³; 494+36, Ben Vuirich granite⁶⁴. *n, o*: 460+25, Mannin Thrust²⁶; 460+7, Oughteraard granite⁶⁵; 462, oldest Connemara muscovite⁴⁸; 500, Cashel intrusion⁶⁶; 515, basal aureole Highland Boundary Fault Zone (B. J. Black, personal communication). *r*: 484, Colmonell Gabbro⁶⁷; 478+8, lava; 506+14, basal aureole; 510, metapyroxenite; 476+14, lava; 501+12, lava (Ballantrae Complex). The time scale (in Myr), given in the second column, was generated iteratively from Caledonian/Appalachian data, slightly modifying a time scale given by W. S. McKerrow.

dipping subduction zone, with⁵ or without² ridge-trench interaction; those appealing to collisions between the Laurentian margin and island arcs with southeastward subduction⁶⁻¹¹, or oceanic plateaus¹² with northwestward subduction; and those involving ophiolite obduction either from the south-east¹³⁻¹⁵ or from the north-west¹⁶. The middle part of the Ordovician period was a time of fundamental but diachronous tectonic change throughout the northwestern Appalachian/Caledonian belt from at least Tennessee to Scotland. The Grampian tract underwent its main structural and metamorphic evolution before Llandeilian times in the British Caledonides (Grampian event), before Caradocian times in Newfoundland (Humberian event) and before late Caradocian times in New York and New England (Taconian event) (Fig. 1). The nature of this event is particularly clear in Newfoundland where the Humberian deformation and metamorphism was driven by the progressive early Ordovician northwestward obduction of an ophiolite nappe^{9,15,17-20}. However, in Newfoundland, the post-Humberian tectonic setting is less obvious, whereas in Scotland the reverse is true; the Grampian event is enigmatic and post-Llandeilian northwestward subduction is evident⁴. Any model of the early Caledonides must account for the following features of the Grampian tract: (1) northwestward progression of structural/metamorphic events with the Moine Thrust Zone as the youngest part of the Highland structure; (2) the gross northwestward vergence of the orogen, emphasized by an overall southeastward-younging from the older Moine Series to the younger Dalradian Series; (3) dominant structural vergence to the north-west with the

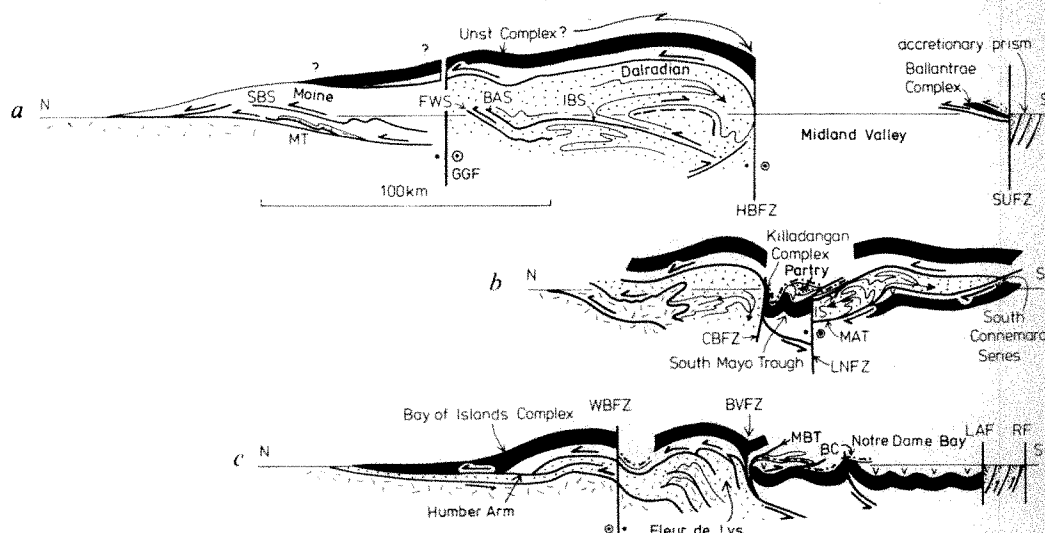
exception of the Tay (and possibly Banff) Nappe; (4) that the Unst Ophiolite Complex (Fig. 1) is east-facing and structurally overlies the Grampian metamorphic complex.

Outline of the Grampian model

Figure 2 summarizes the facies of the Grampian and adjacent tracts, giving isotopic ages and other data and interpretations that are used as a basis for the model. Figure 3 shows three schematic transects of the orogen.

The Humberian Orogeny in Newfoundland resulted from the destruction of a late Precambrian and Ordovician continental shelf/rise by progressive northwestward obduction of an ophiolite nappe (Bay of Islands Complex), whose obduction timing is well constrained from Arenigian to pre-*Climacograptus bicornis*^{17,20}. This coincided almost precisely with the 30-Myr existence of a calc-alkaline volcanic arc (Notre Dame terrane) to the south-east that grew on a late Tremadocian ophiolite foundation and shows a development from an early Arenigian mafic proto-arc to a more silicic late Arenigian/Llanvirnian mature arc. All well-dated ophiolite complexes in western Newfoundland are of Tremadocian age, that is, they were generated only a few million years before the beginning of their obduction. This suggests that the Humberian Orogeny and the initiation, growth and termination of the Ordovician arc resulted from the nucleation of the initial obduction site on a newly-formed ridge-fracture zone system, southeastward subduction resulting from progressive northwestward obduction and, finally, collision of the arc and obduction of its ophiolite foundation and forearc

Fig. 3 Schematic structural sections (drawn to scale) along lines indicated in Fig. 1. BC, Betts Cove; BVFZ, Baie Verte Fault Zone; CBFZ, Clew Bay Fault Zone; FWS, Fort William Slide; GGF, Great Glen; HBFZ, Highland Boundary Fault Zone; IBS, Iltay Boundary Slide; IS, Inisbofin Slide; LAF, Lukes Arm Fault; LNFZ, Lough Nafooy Fault Zone; MAT, Mannin Thrust; MBT, Mings Bight Thrust; MT, Moine Thrust; RF, Reach Fault; SBS, Sgurr Beag Slide; SUFZ, Southern Uplands Fault Zone; WBFZ, White Bay Fault Zone. , Pre-Caledonian basement and Cambro-Ordovician shelf sequences; , Moine/Dalradian/Fleur-de-Lys sequences; white/black areas, ophiolite ultramafics/mafic; V, Ordovician arc volcanics and volcanoclastics; , Ordovician non-marine clastics; — — —, Silurian sediments.



onto the continental margin¹⁸. An Ordovician arc terrane, lying immediately to the south-east of the Grampian tract, occurs intermittently from southern New England to the British Isles (Fig. 1). In Ireland (Fig. 2q), the Ordovician arc shows a mafic to silicic maturation trend, which had begun by mid-Tremadocian times and terminated before the end of the Arenigian. In western New England, arc volcanism continued into early Caradocian times, indicating a diachronous progression of arc development, obduction and collision along-strike towards the south-west. An earliest Llanvirnian intracarbonate disconformity with karsting below and a deepening sequence into shales and easterly derived chromite-bearing turbidites above, is probably the result of flexural loading of the continental margin lithosphere²¹ by the advancing ophiolite sheet; this arching-deepening sequence is younger to the south, being from early to mid-Cardocian age in eastern New York (sub-Chazy/Balmville unconformity).

The contact between the Grampian tract and the arc terrane is a steep zone of ophiolite mélanges and south-east-facing ophiolite slivers (Brompton/Baie Verte/Mings Bight/Clew Bay/Highland Boundary Line^{17,19}), and is interpreted as the suture zone that bounds the Grampian metamorphic complex to the south-east and along which the arc terrane collided with the continental margin^{8,9,17,18}. Within this zone and an immediately adjacent tract comprising the highest Dalradian-Fleur-de-Lys stratigraphy, ophiolite mélanges and ultramafic pods are encased in a matrix of greywacke, black slates and graphitic black schists^{17,22-24} (Killadangan and Achillbeg Complexes). These mélanges are interpreted as a shear carpet formed as ophiolite-derived olistostromes overran and deformed by the northwestward-advancing ophiolite nappe¹⁷. Similar ultramafic pods and slivers occur intermittently within the Grampian Zone in Newfoundland (Pasadena, Coney Head²⁵, Fleur-de-Lys) and in Scotland (Glen Urquhard, Portsoy) and are probably remnant slices of a single supra-Grampian ophiolite sheet tucked into the metamorphics along pinched synclinal slide zones. The Unst Ophiolite Complex is believed to occupy a southeastern root zone position analogous to the Brompton/Clew Bay Zone. The rare occurrences of blueschist in the Grampian Zone are in rocks immediately beneath the proposed ophiolite shear carpet.

Following the development of early Grampian structures with a northwestward vergence, the southeastern part of the Grampian tract and adjacent parts of the Ordovician arc terrane were affected by southward-verging retrocharriage structures following the end of arc volcanism, for example, the D4 Mannin Thrust in Connemara²⁶, the Mings Bight and southeast-verging folds and slide zones of the eastern Burlington Peninsula in New-

foundland, and the rotation of the southern margin of the Tay Nappe into downward-facing attitudes along the Highland Border. In the British Isles, they roughly coincide with the Llandelrian establishment of northward subduction beneath a south-facing arc, along the southern edge of which the Southern Uplands accretionary prism developed. The southeast-verging late Grampian structures may comprise various combinations of shear associated with northward subduction, gravity spreading resulting from D1-D3 crustal thickening and rotation along fault zones at the southern edge of the rising Grampian tract. These southward-verging structures appear to be contemporaneous with, or slightly earlier than, a phase of rapid uplift and denudation of the Grampian tract that began ~460 Myr ago in the British Isles and ~450 Myr ago in Newfoundland and is evidenced by thick clastic sequences (Fig. 2j,p-s) derived from Grampian and Grenville metamorphic sources. The isotopic age pattern of the Dalradian rocks in the British Isles²⁷ indicates a period of rapid uplift, stripping and cooling from 460 to 440 Myr. Uplift was essentially completed by early Silurian times in western Ireland, where Upper Llandoverian strata rest unconformably on the Dalradian (Fig. 2n); uplift was complete by earliest Devonian times south of the Great Glen Fault, where the Lorne lavas lie unconformably on Grampian metamorphics. Of the maximum of ~30 km that was stripped from the Grampian Highlands, 15 km was probably an ophiolite nappe; in Connemara, remnant ultramafic pods at numerous localities along the unconformity between the Dalradian and the Upper Llandoverian suggests the stripping of ~15 km of ophiolite nappe during the mid- and late Ordovician. The Partry Series in the South Mayo Trough is the earliest Grampian molasse and, with earlier Ordovician sediments and volcanics, was folded before the Upper Llandoverian, a phase of shortening which was probably coincident with the mid-Ordovician late D3 to D4 events affecting the Dalradian. Along the southeastern margin of the Grampian Orogen, a short but diachronous lutite lull (Glennamara Slates in western Ireland, *Nemagraptus gracilis*-*C. bicornis* black slates of Newfoundland) immediately followed arc termination and preceded uplift. Thus, we suggest, the final stages of collisional shortening led to a 'flip' in subduction polarity and the beginning of Grampian uplift and denudation.

Grampian ophiolite obduction

All giant ophiolite nappes, including those of Ordovician age in the Appalachian/Caledonian system, are characterized by a very short (<10 Myr) interval between ridge-fracture zone generation and their subsequent obduction detachment^{28,29}, have

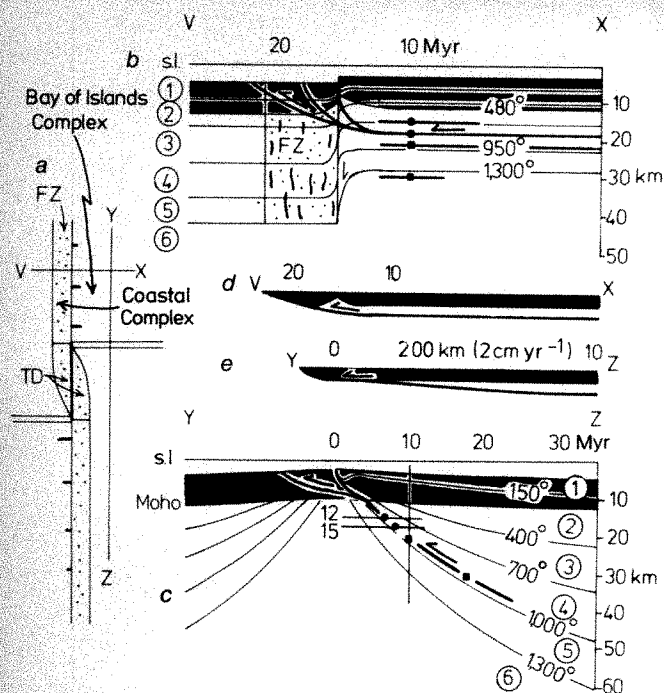


Fig. 4 Nucleation of obduction zone on a ridge transform plate boundary and its fracture zone extensions. *a*, Plan of ridge/fracture zone indicating lines of section in *b* and *d* (VX) and *c* and *e* (YZ). TD, transform domain; FZ, polyphase-deformed rocks of transform/fracture zone. *b*, Section across a fracture zone with a contrast between 10 and 20 Myr lithosphere showing detachment of a 15-km-thick ophiolite sheet with a frontal portion of fracture zone rocks. FZ, fracture zone; black areas, mafic rocks; white areas, ultramafic rocks. 1, Brittle; 2, semi-brittle; 3, low-temperature ductile; 4, high-temperature ductile; 5, plastic; 6, low-velocity zone. ■, Range of pressure estimates from basal aureole³⁴; ●, bases of 12-km and 15-km-thick ophiolite sheets. *c*, Section across a ridge showing detachment of an ophiolite sheet along the 950 °C isotherm (solid ■ and ● as in *b*). *d*, True-scale section VX. *e*, True-scale section YZ generated at a half-spreading rate of 2 cm yr⁻¹.

inverted high-temperature metamorphic sole aureoles^{28,29}, initial detachment thicknesses of between 10 and 15 km²⁹, commonly carry primitive calc-alkaline arc volcanics, and may have oceanic fracture zone assemblages along the leading obduction edge^{18,30,31}. This is consistent with the detachment of a slice of oceanic crust and mantle, almost immediately after its generation, along a ductile zone at or near an accreting plate boundary crest or fracture zone, or a combination of both (Fig. 4). The earliest, highest temperature part of the obduction aureole in the Bay of Islands Complex was generated at ~950 °C (refs 32, 33) at the base of a 15-km ophiolite slab, a temperature/depth ratio appropriate for oceanic lithosphere <10 Myr old (Fig. 4c). Ophiolite obduction is easier shortly after generation because the 950 °C potential detachment surface becomes progressively deeper as the lithosphere ages (Fig. 4c). It is less clear what causes ridge → obduction zone conversion. Opening and closing of back and intra-arc basins has been suggested²⁸ but this is unlikely for Grampian ophiolites because they predate immediately volcanic arc development (Fig. 2f-j). Three principal mechanisms seem possible. First, changes in relative motion and intraplate and plate boundary stress patterns may result from regional or global plate boundary reorganization. Obduction by such a cause is likely to be roughly synchronous along great strike lengths of a continental margin, to predate arc development in the region and not to involve substantial syn-obduction rotations.

A second mechanism is related to an evolving trench/transform triple junction such as the Pacific/North American/Farallon junction on the early Tertiary Californian borderland. Flakes

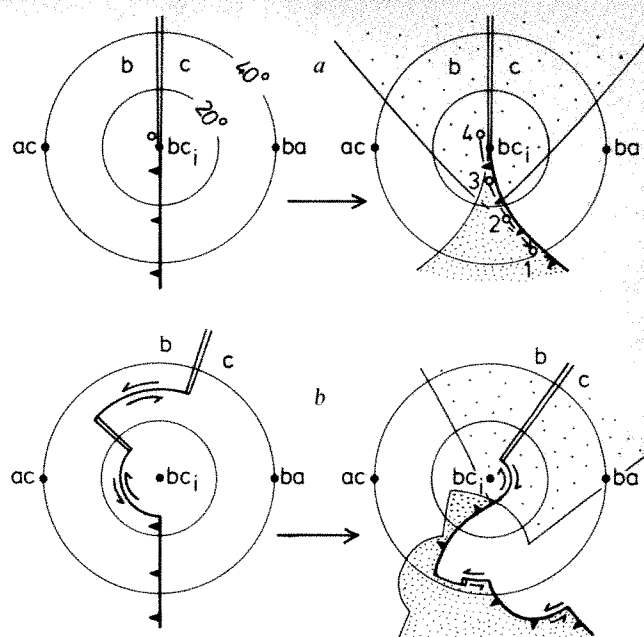


Fig. 5 Central portion of lower hemisphere equal-angle stereographic projections showing two plate boundary geometries that allow a close sequential relationship between ophiolite generation (double line) and beginning of obduction (barbed line). Plates *b* and *c* rotate around poles *ba* and *ac* respectively, which are fixed in the frame of reference of the projection and plate *a*. *bc_i*, The instantaneous rotation pole that describes the relative motion of plates *b* and *c*. ■, Area of plate *c* subducted beneath plate *b* after 60° of rotation of plates *b* and *c* around *ba* and *ac*, respectively; □, area of plates *b* and *c* generated and not subducted, 1-4 represent localities in order of decreasing age at which ophiolite generation is shortly followed by obduction.

of newly-formed ophiolite could continuously transfer to a transform segment that had just been a subduction zone. This obduction mechanism would involve rather small flakes with substantial rotations and would terminate rather than precede volcanic arc activity. A third mechanism results from an inevitable consequence of plate boundary evolution—the progressive change in position of plate boundaries relative to the poles of rotation that describe their relative motion. Where ridge-fracture zone systems pass through or lie close to instantaneous rotation poles, a progressive change from plate divergence to convergence is possible³⁴ (Fig. 5a,b). This mechanism must generate diachronous change from spreading to obduction and is unlikely to cause substantial syn-obduction rotations.

The first and third mechanisms seem likely for large-scale ophiolite obduction, where ophiolite generation generally precedes arc growth. An example of the first mechanism may be the roughly synchronous Neotethyan late Cretaceous obduction from Cyprus to the Himalayas. The progressively later growth and demise of the Ordovician arc along-strike to the south-west within the Appalachian/Caladonian Orogen is best explained by the third mechanism.

Obduction detachment at a fracture zone^{18,30} produces a different configuration from detachment at a ridge crest^{28,29}. Fracture zone obduction (Fig. 4b,d) will involve an ophiolite nappe of approximately uniform across-strike but varying along-strike thickness, with a sheeted dike trend normal to the detachment line. Frontal nappe assemblages will consist of mafic/ultramafic assemblages generated along a fracture zone with complicated polyphase structural and magmatic histories, which form the principal obduction aureole protoliths. Assuming an obduction detachment surface dip of 5° and a converge rate of 2 cm yr⁻¹, the first gabbroic protoliths near the base of the oceanic crust will take 4 Myr to reach the highest aureole temperature and the uppermost oceanic crust will take 8 Myr.

By contrast, ridge crest obduction will generate an ophiolite nappe with approximately constant along-strike thickness, but thinning in the direction of obduction with a sheeted dike trend parallel with the detachment line. Frontal nappe assemblages will consist of newly generated oceanic crust and mantle and will form the earliest obduction aureole protoliths with temperatures as high as 1,200 °C at pressures as low as 2 kbar.

In Newfoundland, the evidence favours ophiolite detachment along a north-east-striking fracture zone (Coastal Complex), a continuation of the dextral transform portion of a long transform/short ridger system approximately parallel with, and a few hundred kilometres from, the early Ordovician continental margin³⁰. The obduction aureole comprises the first stages of a progressive northwestward and downward Arenigian nappe-stacking sequence that culminated with the emplacement of the whole Grampian nappe assemblage onto the platform during the mid-Ordovician. The progressive diminution of aureole temperature and pressure (Fig. 6f) indicates cooling and thinning of the ophiolite sheet during obduction. In a compressional regime, the thinning is unlikely to be tectonic in origin, nor can it have been due to syn-obduction erosion of a planar ophiolite sheet because the highest parts of the oceanic crust are preserved in the Bay of Islands Complex. Also, the earliest stages of thrusting of a 15-km sheet of 10 Myr ophiolite across a 20-Myr oceanic lithosphere would involve wholly submarine obduction with water depths of ~3 km. Even during the earliest stages of late D1/D2 progressive stacking of Dalradian continental margin sediments, shortening was occurring in a zone of thin continental crust and, therefore, emergence would have taken place only when the crust and sediments were restacked to above 30 km¹².

This event is witnessed by the Llanvirnian Crabb Brook³⁵ ophiolitic clastics (Fig. 2e) that rode on the Bay of Islands ophiolite complex. Thinning of the ophiolite nappe was probably mainly due to D3 buckling and erosional stripping, accounting for steeply dipping complete ophiolite sections transected by the final obduction thrust (Fig. 6d). During this early Arenigian early obduction phase, the obduction aureole and the highest Dalradian rocks in ophiolitic wildflysch sequences, such as those of Clew Bay³⁶, assume great significance because they probably record a direct relationship between structure and fabric and the obduction slip direction, which in turn is likely to be, more or less, the direction of relative motion of the plate. The aureole, in particular, will record the very earliest convergence slip direction following and perhaps progressively changing from the ridge transform direction, which will be preserved in the frontal portion of the ophiolite sheet and, more obscurely, in ophiolitic blocks in the highest wildflysch. Complicated structural sequences in mafic protoliths in metamorphic belts with younger structural sequences must be viewed, therefore, with extreme caution; they may represent obducted fracture zone rocks and not portions of an older crystalline basement. Systematic studies of early obduction structures have not been undertaken and are of paramount importance in attempts to relate structural geology to plate tectonics.

As D3 shortening progressed, the regional plane strain that had characterized D1 and D2 led to a progressive loss of the relationship between slip vector and fabric and to more complicated regional strains, partly driven by body forces involving flaking, wedging and rotation, with axial extensions and consequent strike-slip motions in a locally 70 km-thick crust. D3 shortening, with complexities probably partly due to the irregularities of the Laurentian rifted margin, choked the obduction zone, terminated the arc and led to D4 rotation²⁶ and retrocharriage and to northward subduction beneath, and uplift of, the Grampian tract (Fig. 6d,e). Mafic intrusions occur in several parts of the Grampian tract (Cashel Suite in Connemara, Newer Gabbros in Scotland) and predate D3. If they are the roots of arc volcanics³⁷, this model is invalidate, at least for the British Isles, because they would imply northward subduction during the Arenigian. They may, however, have been

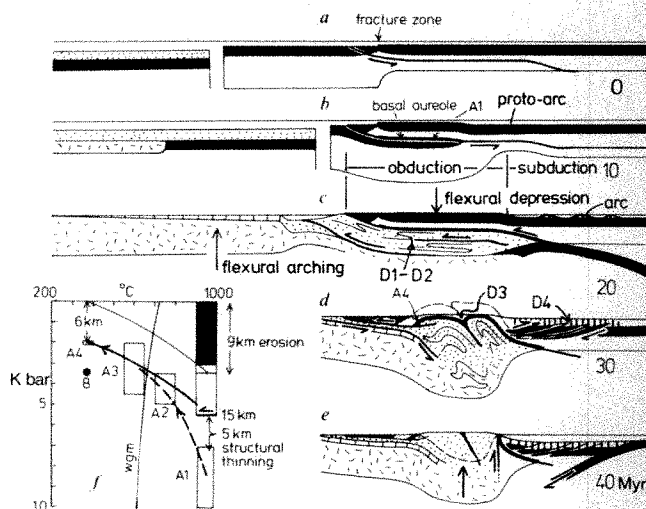


Fig. 6 a-e, Approximately true-scale schematic sections to account for the Grampian Orogeny, illustrating the progressive obduction of an ophiolite sheet across, and the sequential stacking of, a continental rise and continental shelf, the shortening and thickening of the continental margin rocks and the consequent flip in subduction polarity; for symbols see Fig. 3. f, Pressure-temperature plot of rocks of the basal aureole³². A1, two-pyroxene amphibolites; A2, amphibolites with trondhjemitic partial melts; A3, garnet-epidote amphibolites; A4, actinolite-greenschists; wgm, wet granite melting curve; B, crossite-bearing metamorphics.

developed in strike-slip pull-aparts or by delamination of the lithosphere³⁸ during shortening.

Displaced terranes and the closure of Iapetus

The Bay of Islands fracture zone model³⁰ implies a right-lateral component of Tremadocian/Arenigian relative plate motion across the Grampian Zone. Post-Llandeilian relative motion, however, until the late Silurian to mid-Devonian diachronous closure of Iapetus, involved a substantial component of left-lateral displacement. Very large sinistral late Devonian-Carboniferous displacements have been suggested^{39,40} to have occurred along the Appalachian/Caledonian chain, but the geological evidence clearly shows Carboniferous motion to have been right-lateral^{12,41}. We believe that all sinistral motion occurred between Caradocian and mid-Devonian times, based on the following evidence. The Great Glen/Leannan Fault and other roughly parallel faults moved during the early Devonian⁴² and probably have a total cumulative offset of a few hundred kilometres. Extensional bends in associated shear zones were critical in controlling the emplacement of granite plutons⁴³. The Highland Boundary Fault truncates the Dalradian terrane and juxtaposes it with the Midland Valley, which appears to consist of a Grenville basement stretched and underplated by a mafic lower crust during the latest Precambrian (B. J. Upton, personal communication) with superposed early Ordovician ophiolite obduction, a terrane very similar to the Long Range of southwest Newfoundland. Their original contiguity implies a sinistral displacement of at least 1,000 km which probably occurred from late Silurian to the end of the early Devonian, giving a slip rate of ~5 cm yr⁻¹. This large sinistral motion would account for the entrainment of a wide variety of Ordovician and Silurian rocks along the Highland Border⁴⁴. This further implies that the clastic source of the Southern Uplands accretionary prism, which must have travelled at least as far as the Midland Valley, cannot have been the Scottish Highlands, a notion supported by the fact that the Lower Devonian rocks of the Midland Valley have a source other than the Scottish Highlands⁴⁵.

In western Ireland, the Dalradian rocks of Connemara are in an anomalous position to the south of the Clew Bay Ophiolite Zone and the Ordovician volcanic rocks of the South Mayo Trough^{46,47}. The latter cannot have been thrust across the

Dalradian because their very preservation means that they escaped the large uplifts and denudations (>15 km) suffered by the Grampian metamorphics and hence cannot be underlain by Dalradian rocks. Upper Llandoveryan strata rest unconformably across a concealed contact between the Ordovician rocks of the South Mayo Trough and the Connemara Dalradian where the Lough Nafuoey Fault Zone was a major controlling influence on Arenigian volcanism and sedimentation⁴⁸. This fault zone is interpreted here as a major post-Llandeilian, pre-Upper Llandoveryan strike-slip fault along which Connemara was displaced from the set by ~500 km. Lastly, early Devonian terminal closure of Iapetus in north-central Newfoundland had a strong sinistral component as evidenced by sets of sinistral Riedel faults in New World Island between the Lukes Arm and Reach Faults. The sum of all these post-Grampian sinistral motions is at least 1,800 km and had the effect of severely modifying, by strike-slip omission and repetition, the Grampian tract and its post-Llandeilian subduction-accretion neighbouring terrane to the south, and may have been responsible also for substantial pre-collisional modification to the southern margin of Iapetus such as the truncation of the Lake District forearc region⁴⁹. Perhaps the northern margin of Iapetus between Llandeilian and late Silurian times was similar to the Mesozoic-Cenozoic western margin of North America where complex plate interactions were driven by roughly strike/slip motion between the large plates.

Conclusions

The pre-Grampian margin of the Laurentian Shield in the Appalachians was a continental shelf-rise generated by latest Precambrian continental separation. Continental separation was heralded by the eruption of the Tayvallich and Lighthouse Cove mafic lavas. The Dalradian rocks were evidently laid down in a basin bounded to the south by a horst³. The pre-Grampian right-lateral phase of offshore ridge/transform tectonics may have been responsible for the removal of this crystalline horst (perhaps now the basement of the Ammonoosuc Zone in western New England), much as the opening of the Gulf of California is transporting a narrow Baja continental sliver northwards to open up the transform continental borderland of Mexico. The offshore ridge/transform boundary was converted, almost immediately and probably diachronously, along the length of the Appalachian/Caledonian Orogen, into an obduction zone during Tremadocian-early Arenigian times. It was the progressive northward obduction of a 15-km ophiolite sheet that stacked a subadjacent Dalradian nappe pile, to the south of which grew an Arenigian volcanic arc on a Tremadocian ophiolite foundation. Grampian crustal shortening was below sea level for a substantial part of the early Ordovician until rapid D3 shortening and crustal thickening led to a flip in subduction polarity, the beginning of southward-verging subduction accretion and rapid uplift and stripping of the Grampian tract. The ophiolite nappe is preserved in the steep suture zone between the arc and the Dalradian terrane and where it reached the platform. In the uplifted and denuded axial part of the Grampian Zone, it is preserved only in high-level slide zones in lower grade regions.

The obduction model proposed here may be a template for the evolution of most Barrovian mountain belts of Grampian type that evolve prior to terminal oceanic closure. Major ophiolite obduction seems to occur long before continental collision and emphasizes the observation of Hess⁵⁰ that the great Alpine-type ultramafic belts were generated during the first major deformational events in the mountain belt that contains them. This massive scale of obduction appears to result from ridge-fracture zone → obduction zone conversion and may be significant with regard to global plate tectonics. As in the early Ordovician, a similar period of widespread obduction occurred in mid- and late Cretaceous times and was also temporally coincident with blueschists, arc activity, seamount-continental margin collision, continental margin orogeny and widespread platform carbonate signalling a high sea level and rapid subduction¹².

The post-Grampian sinistral motion of almost 1,800 km has

implications for the relationship between the Grampian margin of the Laurentian Shield, the Avalonian terrane and the Baltic Shield (Fig. 1). If this relative motion were carried along-strike between Greenland and Scandinavia, it would imply that the south-east-verging Scandinavian Caledonides were opposite Newfoundland and Ireland in immediately post-Grampian times. As a more plausible explanation, we suggest that a major oceanic triple-junction complex existed in the North Sea⁵¹ between the Scandinavian, British and North German Caledonides and that subduction along the latter absorbed a large part of the Avalonian-Laurentian relative motion. Considerable differences exist between the Grampian tract in the Appalachians and Caledonides. In the Appalachians, a narrow zone of Grampian thrusting and ophiolite obduction was completed by mid-Ordovician times and the Connemara Dalradian was stripped by late Llandoveryan times. In Scotland, the Grampian Zone is wider and thrusting continued into the Silurian in the Moine terrane⁴². A possible explanation is that a D4 subduction polarity change following D1-D3 obduction was followed shortly by Silurian continental collision between the Laurentian and Baltic Shields and by progressive oceanic closure, with a strong sinistral component, culminating in late Silurian to mid-Devonian collision between Avalonia and a semi-consolidated Laurentian/Baltic continent. This collision induced Devonian sinistral strike-slip between the Laurentian and Baltic Shields (Great Glen phase). Before terminal closure of Iapetus, major strike-slip faulting occurred along the Grampian borderland of Laurentia. Of the 1,800 km of Avalonia-Laurentia relative motion, about 500 km was absorbed by late Silurian-early Devonian shortening in the North German/Polish Caledonides, and the remainder accounted for the Devonian left-lateral displacement of Scandinavia relative to Laurentia.

We acknowledge discussions with D. H. W. Hutton and N. J. Soper, who in complementary work have reached similar conclusions about separate Laurentian-Baltic and Laurentian-Avalonian collisions. We thank W. S. McKerrow and H. Williams for critical reviews.

Received 5 July; accepted 21 September 1984.

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Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence

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Cloning and sequence analysis of cDNA for the Electrophorus electricus electroplax sodium channel indicate that this protein, consisting of 1,820 amino acid residues, exhibits four repeated homology units, which are presumably oriented in a pseudosymmetric fashion across the membrane. Each homology unit contains a unique segment with clustered positively charged residues, which may be involved in the gating structure, possibly in conjunction with negatively charged residues clustered elsewhere.

THE sodium channel is a membrane protein that mediates the voltage-dependent modulation of the sodium ion permeability of electrically excitable membranes^{1,2}. The ionic basis for the generation of action potentials has been well understood³. The initial increase and subsequent decrease in sodium ion permeability during an action potential implies that the sodium channel can exhibit at least three distinct functional states: resting (closed), activated (open) and inactivated (closed). A variety of pharmacological agents, including neurotoxins, affect the complex function of the sodium channel⁴. The use of binding assays based on the high specificity of such neurotoxins has permitted purification of the sodium channel from the electric organ of the eel *Electrophorus electricus*⁵⁻⁷ as well as from rat brain⁸, rat skeletal muscle⁹ and chick cardiac muscle¹⁰. It has been reported that the *Electrophorus* sodium channel⁷ and its avian counterpart¹⁰ consist of a single polypeptide of molecular weights 260,000-300,000 and 230,000-270,000, respectively, whereas its mammalian counterparts^{8,9} contain, in addition to the corresponding large polypeptide, two or three smaller polypeptides of molecular weights 37,000-45,000. The purified eel and rat sodium channel preparations have been shown to be functional when reconstituted into phospholipid vesicles¹¹⁻¹⁴.

To understand the molecular mechanisms underlying the selective ion transport and voltage-dependent gating operated by the sodium channel, it is essential to elucidate the primary structure of the constituent protein. Using recombinant DNA techniques, we have now cloned DNA sequences complementary to the messenger RNA coding for the sodium channel protein of *El. electricus* electroplax. Nucleotide sequence analysis of the cloned cDNA has revealed the complete amino acid sequence of this polypeptide.

Partial amino acid sequence

The initial approach used to clone cDNA sequences for the sodium channel protein was to combine immunological screening of a cDNA expression library with identification of the

isolated clones by comparison with the partial amino acid sequences determined by peptide analysis. The sodium channel protein from *El. electricus* electroplax was solubilized with Lubrol-PX and purified by chromatography on DEAE-Sephadex and gel filtration on Sepharose 6B (refs 5, 6), followed by gel filtration on Sepharose 4B in denaturing conditions⁷ and gel permeation HPLC (see Fig. 1 legend). This procedure yielded a homogeneous preparation (Fig. 1A). The purified protein was digested with trypsin, and the resulting peptides were fractionated by reverse-phase HPLC (Fig. 1B). After rechromatography (Fig. 1C), five peptide fractions (fractions Ib, IIa, IIb, IVa and Vb) were isolated and analysed for amino acid sequence with the use of a gas-phase sequencer¹⁵. Six peptide sequences were thus determined (Fig. 1D); fraction Ib proved to contain two peptides. Attempts to determine the amino-terminal sequence of the whole sodium channel protein by Edman degradation were unsuccessful.

Isolation of cDNA clones

A library of cDNA clones was constructed with poly(A)-containing RNA from *El. electricus* electroplax as template and the plasmid expression vector pUC8 (refs 16, 17). Screening of the cDNA library¹⁷ with rabbit antiserum to the purified *El. electricus* sodium channel protein yielded four immunologically positive clones from $\sim 8 \times 10^4$ transformants. Plasmid DNA was isolated from the four clones, and the cDNA insert of each clone was subjected to blot hybridization analysis¹⁸ with a synthetic oligodeoxyribonucleotide probe containing all possible cDNA sequences predicted from the partial amino acid sequence Thr-Phe-Phe-Glu-Pro of the tryptic peptide Vb (see Fig. 1D, e); the third position of the proline codon was not included. Only one of the four clones, pSCH1 (see Fig. 2), carried a hybridization-positive cDNA insert. Nucleotide sequence analysis¹⁹ of this cDNA insert revealed that clone pSCH1 contained a 426-base pair (bp) cDNA insert including the coding sequence for the whole known amino acid sequence

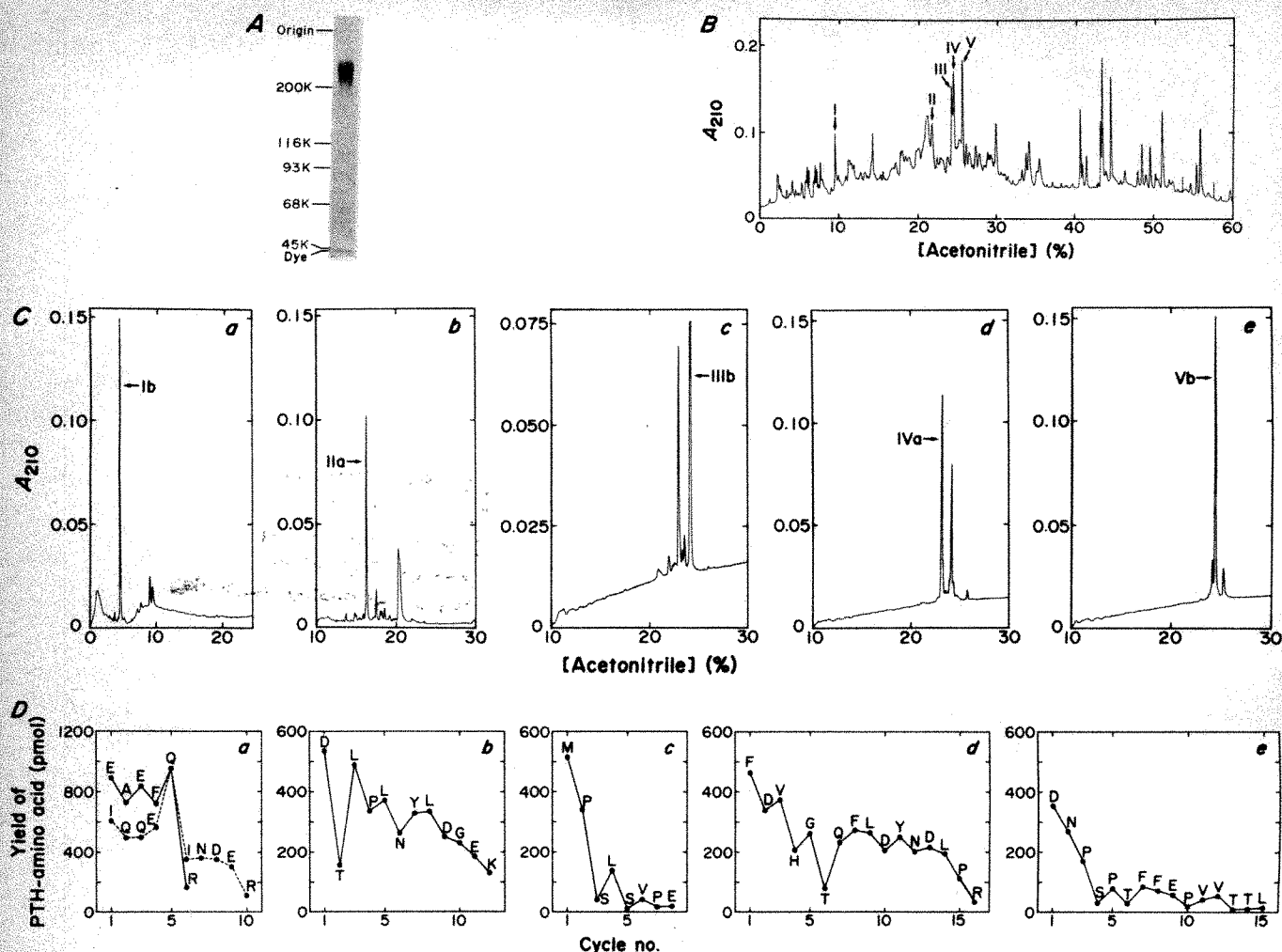


Fig. 1 Purification and partial amino acid sequence analysis of the *El. electricus* sodium channel protein. **A**, Electrophoretic analysis of the purified protein on an SDS-6% polyacrylamide gel¹³. The size markers used were chicken ovalbumin [molecular weight (M_r), 45,000 (45K)], bovine serum albumin (M_r , 68K), rabbit muscle phosphorylase *b* (M_r , 93K), *Escherichia coli* β -galactosidase (M_r , 116K) and rabbit myosin heavy chain (M_r , 200K); bromophenol blue served as tracking dye. The polypeptides were stained with Coomassie brilliant blue. **B**, Reverse-phase HPLC of tryptic peptides. The tryptic digest was directly loaded onto a Chemcosorb 3 μ C₁₈-H column (8 $\times$ 75 mm; Chemco) equilibrated with 0.1% trifluoroacetic acid, and a linear gradient of 0–60% acetonitrile was applied over 80 min at a flow rate of 2 ml min⁻¹; a Hitachi 635 HPLC system with a solvent programmer was used. The eluate absorbance at 210 nm was recorded with a Hitachi 41 UV monitor. Five fractions (I–V) corresponding to absorbance peaks were collected. **C**, Second reverse-phase HPLC. Fractions I, II, III, IV and V (**a**, **b**, **c**, **d** and **e**, respectively) were further purified by reverse-phase HPLC on a Novapak 5 μ C₁₈ column (3.9 $\times$ 150 mm; Waters). The column was equilibrated with 0.1% ammonium acetate buffer, pH 6.0 (**a**), 0.1% ammonium acetate buffer, pH 6.0, containing 10% acetonitrile (**b**) or 10 mM ammonium formate buffer, pH 4.0, containing 10% acetonitrile (**c–e**), and a linear gradient of 0–60% (**a**), 10–60% (**b**) or 10–50% acetonitrile (**c–e**) over 60 min was used at a flow rate of 1 ml min⁻¹. Peak fractions (Ib, IIa, IIIb, IVa and Vb) were collected. **D**, Sequence analysis of peptides. Fractions Ib (**a**), IIa (**b**), IIIb (**c**), IVa (**d**) and Vb (**e**) were subjected to amino acid sequence analysis with a gas-phase sequencer¹⁵ (model 470A, Applied Biosystems). Phenylthiohydantoin (PTH)-amino acids were analysed by HPLC at 50 °C using an Ultrasphere ODS column (4.6 $\times$ 250 mm; Altex) with a three-solvent gradient (acetonitrile, water and 40 mM sodium acetate buffer, pH 5.6) at a flow rate of 1 ml min⁻¹. The yield of PTH-amino acids at each cycle of Edman degradation is shown; the one-letter amino acid notation is used. Fraction Ib was a mixture of two peptides, whose sequences were assigned as indicated, assuming that glutamine is shared by the two peptides at the fifth amino acid position.

Methods: Approximately 2 mg of the partially purified sodium channel protein (Sephacose 6B fraction^{5,6}), derived from about 200 g of *El. electricus* electric organ (World Wide Scientific Animals), was further purified by gel filtration on Sepharose 4B in denaturing conditions⁷. Fractions enriched in sodium channel protein (derived from ~600 g of electric organ) were combined and alkylated at 25 °C for 30 min with 0.1 M iodoacetamide in the presence of 0.13 M 2-mercaptoethanol, 0.1% SDS and 0.4 M Tris-HCl buffer, pH 8.0. After desalting with a Biogel P-100 column (1.5 $\times$ 20 cm) equilibrated with 0.02% SDS, the sodium channel protein was finally purified by gel permeation HPLC on a TSK 4000 column (7 $\times$ 600 mm; Toyo Soda) using 50 mM sodium phosphate buffer, pH 7.5, containing 0.1% SDS at a flow rate of 0.5 ml min⁻¹; the yield was ~300 μ g. The purified material was dialysed against water, lyophilized and dissolved in 90 μ l of 0.1 M ammonium bicarbonate containing 10 mM CaCl₂. TPCK-treated trypsin (Worthington) was added at a sodium channel protein/trypsin ratio of 100. After incubation at 37 °C for 12 h, a further identical aliquot of TPCK-treated trypsin was added, and the incubation was continued for additional 12 h.

of the tryptic peptide Vb. When total RNA from *El. electricus* electroplax was subjected to blot hybridization analysis²⁰ with the cDNA insert of each of the four clones as probe, an RNA species of ~8,000 nucleotides was hybridizable not only with clone pSCH1 but also with another clone, pSCH4 (see Figs 2, 4); no hybridizable RNA was detected with the two remaining clones. This suggested that both pSCH1 and pSCH4 carried a cDNA sequence for the sodium channel protein.

To clone longer cDNA sequences, we next constructed a cDNA library, using poly(A)⁺ RNA from *El. electricus* electroplax and the plasmid vector elaborated by Okayama and Berg²¹. Screening of this library²² by hybridization with the combined cDNA inserts of clones pSCH1 and pSCH4 yielded 14 hybridization-positive clones from ~2 $\times$ 10⁵ transformants. All 14 clones

hybridized with the pSCH1 probe, whereas only 6 of them hybridized with the pSCH4 probe. Clone pSCH14, which apparently harboured the largest cDNA insert among the 14 clones, contained the whole cDNA sequence carried by pSCH1, but did not extend to the 5' end of the cDNA carried by pSCH4 (see Fig. 2). Therefore, additional ~1 $\times$ 10⁶ transformants from the same Okayama-Berg library were screened with a hybridization probe derived from the 5'-terminal portion of the cDNA insert of pSCH4. Thus, five hybridization-positive clones were isolated. One of these clones, pSCH50, which apparently contained the largest cDNA insert among them, extended beyond the 5' end of the cDNA insert of pSCH4 (see Fig. 2).

Because clone pSCH50 apparently did not carry the entire protein-coding sequence, however, additional clones harbouring

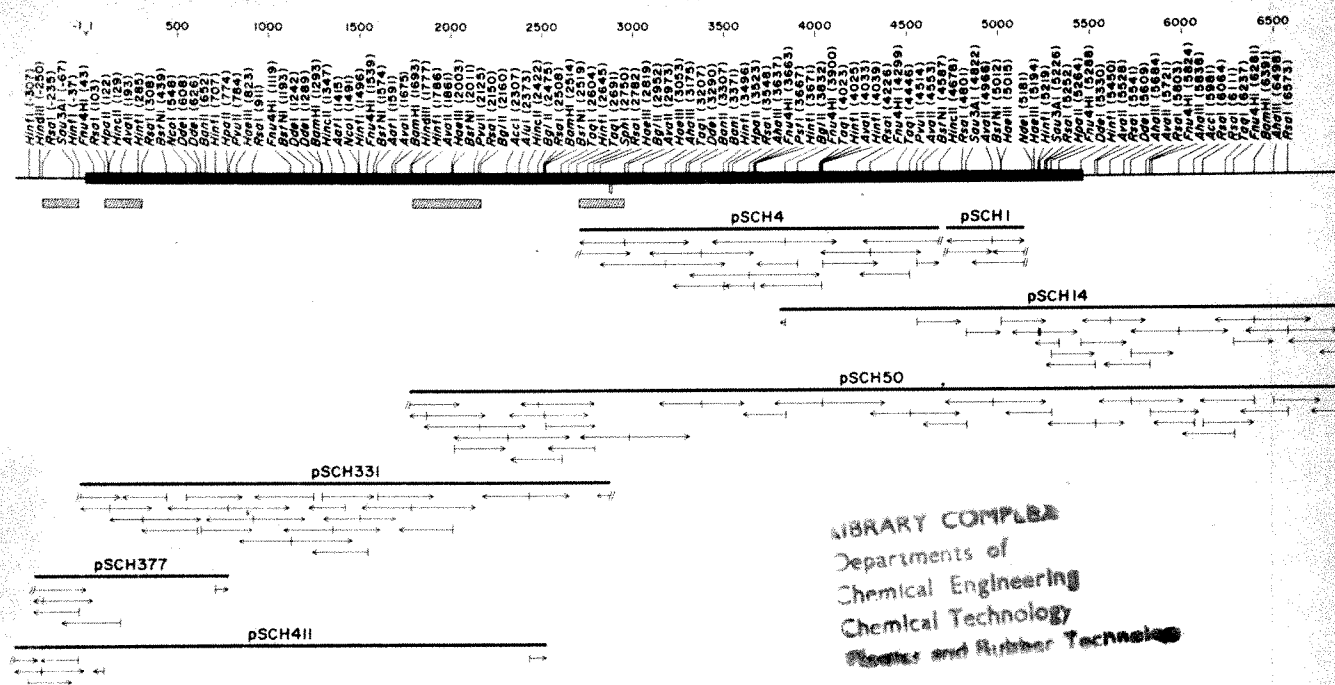


Fig. 2 Strategy for sequencing cloned cDNA encoding the *EL electricus* sodium channel protein. The restriction map displays only relevant restriction endonuclease sites, identified by numbers indicating the 5'-terminal nucleotide generated by cleavage (for the nucleotide numbers, see Fig. 3). The poly(dA)·poly(dT) tract and the poly(dG)·poly(dC) tails are not included in the restriction map. The protein-coding region is indicated by a closed box, the sequence used for specific priming of reverse transcription by a small open box and the sequences used as hybridization probes for selecting clones by hatched boxes. The direction and extent of sequence determinations are shown by horizontal arrows under each clone used; the sites of 5'-end labelling are indicated by short vertical lines at the end of arrows. The slash marks at the end of arrows mean that the site of 5'-end labelling was located on the vector DNA^{21,46,47} (*Hpa*II, *Pst*I or *Pvu*II), on the linker DNA (*Eco*RI or *Sal*I) or at the boundary between the 3'-non-coding sequence and the poly(dA)·poly(dT) tract (*Aha*III) as follows: *Eco*RI (left marks) and *Sal*I (right marks) for pSCH1 and pSCH4; *Aha*III (upper right mark) and *Pvu*II (lower right mark) for pSCH14; *Pst*I (left mark) and *Pvu*II (right mark) for pSCH50; *Pst*I for pSCH331 and pSCH377; *Hpa*II for pSCH411.

Methods: Total RNA was extracted⁴⁸ from *EL electricus* electric organs, and poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography⁴⁹. The procedures used for the construction of the cDNA expression library and for the immunological screening of transformants were essentially the same as described by Helfman *et al.*¹⁷. Using 15 µg of poly(A)⁺ RNA, we obtained 500 ng of cDNA, whose internal *Eco*RI sites were blocked by methylation. The cDNA library constructed by ligation to *Eco*RI/*Sal*I-cut pUC8 plasmid¹⁶ gave ~3,000 ampicillin-resistant transformants per nanogram of cDNA when the efficiency of transformation with supercoiled pUC8 DNA was 3×10^4 ng⁻¹. The library transformed to *E. coli* DH1 (ref. 50) and plated on nitrocellulose filters was screened by incubating the filters first with rabbit antiserum to the purified *EL electricus* sodium channel protein⁵¹ and then with ¹²⁵I-protein A (NEN); the antiserum had been preabsorbed at 4 °C for 5 h with a sonicated lysate prepared from the parental bacterial strain DH1, which carried pUC8 plasmid. The oligodeoxyribonucleotide probe used for testing the four immunologically positive clones was a mixture of 32 tetradecaners, 5'-GGTCAAGAAAGT-3', synthesized⁵² as two pools containing A/T or G/C at the twelfth position, where N denotes a mixture of A, T, G and C. The blot hybridization was carried out by the procedure of Southern¹⁸ with the probe labelled with ³²P at the 5' end. RNA blot hybridization analysis was performed by the procedure of Thomas²⁰. Clones pSCH1 and pSCH4 were thus isolated. For the isolation of clones pSCH14 and pSCH50, a cDNA library was constructed by the method of Okayama and Berg²¹, using 10.2 µg of poly(A)⁺ RNA and 4.2 µg of the vector-primer DNA. *E. coli* HB101 (ref. 53) was used for transformation⁵⁴, and ampicillin-resistant transformants were screened²² by hybridization at 60 °C with the following DNA fragments: all hybridization probes were labelled by nick-translation⁵⁵ with [α -³²P]dCTP unless otherwise stated. The first screening was carried out with an equimolar mixture of the *Eco*RI/*Sal*I-excised cDNA inserts of pSCH1 and pSCH4, which carried residues 4,716–5,141 and residues 2,703–4,674, respectively, and the second screening with the 257-bp fragment (excised from pSCH4) extending from the *Eco*RI site flanking the cDNA insert to the *Bgl*II (2,952) site. For the isolation of clones pSCH331, pSCH377 and pSCH411, the synthetic oligodeoxyribonucleotide primer 5'-CATGCTTCGTGATC-3', complementary to residues 2,872–2,885, was elongated by reverse transcription of the mRNA. Single-stranded cDNA was synthesized using 1 nmol of this primer and 200 µg of electroplax poly(A)⁺ RNA (denatured by methylmercuric hydroxide) as template and was converted to double-stranded cDNA, which was cloned in pBR322 plasmid by inserting it into the *Pst*I site using poly(dG)·poly(dC) homopolymeric extensions; the procedures used were as described previously⁵⁶. In the first screening, ~1,000 hybridization-positive colonies were obtained from ~1 × 10⁶ tetracycline-resistant transformants, using the *Hinf*I (1,786)–*Bgl*II (2,160) fragment derived from pSCH50 as probe. Forty-eight clones, selected randomly, were isolated, and clone pSCH331 harboured the longest cDNA insert among them. In the second screening, 65 hybridization-positive colonies were obtained from ~5 × 10⁵ transformants, using the *Rsa*I (103)–*Rsa*I (308) fragment derived from pSCH331 as probe. Among the 24 clones selected randomly, clone pSCH377 carried a cDNA insert extending most upstream. In the third screening, where the filters used in the second screening were re-used, 25 additional hybridization-positive clones were isolated, using the *Rsa*I (–235)–*Hinf*I (–37) fragment derived from pSCH377 as probe. Among them, clone pSCH411 carried a cDNA insert extending most upstream.

cDNA sequences for a region further upstream were isolated as follows. A specific oligodeoxyribonucleotide primer corresponding to a sequence found near the 5' end of the cDNA insert of clone pSCH4 was elongated by reverse transcription of the mRNA, and the resulting cDNA transcripts were cloned into the plasmid pBR322 and screened by hybridization with appropriate restriction fragments derived from cDNA clones isolated earlier. Thus, clones pSCH331, pSCH377 and pSCH411, whose cDNA inserts all extended beyond the translational initiation site, were obtained (see Fig. 2).

Nucleotide sequence analysis of the cDNA clones was carried out by the procedure of Maxam and Gilbert¹⁹ according to the strategy indicated in Fig. 2. Experimental details for the isolation of the cDNA clones are given in Fig. 2 legend.

cDNA and protein sequences

Figure 3 shows the 7,230-nucleotide sequence (excluding the poly(dA) tract) of the cDNA encoding the *EL electricus* sodium channel protein, determined using clones pSCH1, pSCH4, pSCH14, pSCH50, pSCH331, pSCH377 and pSCH411. No nucleotide difference was observed among the cDNA sequences determined with the individual clones, except that the polyadenylation site differed by four nucleotides between clones pSCH14 and pSCH50 (see below). All of the six partial amino acid sequences determined by peptide analysis (see Fig. 1D) were found to be encoded by the cDNA sequence in the same reading frame (amino acid residues 420–425, 432–441, 861–872, 1,596–1,611, 1,677–1,691 and 1,774–1,781). By using this reading

Fig. 3 Nucleotide sequence of cloned cDNA encoding the *EL electricus* sodium channel protein. The nucleotide sequence was determined with clones pSCH1, pSCH4, pSCH14, pSCH50, pSCH331, pSCH377 and pSCH411. Nucleotide residues are numbered in the 5' to 3' direction, beginning with the first residue of the ATG triplet encoding the initiative methionine, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers; the number of the nucleotide residue at the right end of each line is given. The deduced amino acid sequence of the sodium channel protein is shown above the nucleotide sequence, and amino acid residues are numbered beginning with the initiative methionine. Residue 6,846 in the cDNA insert of clone pSCH14 and residue 6,842 in that of clone pSCH50 are followed by a poly(dA) tract, which is connected with the vector DNA sequence.^{21,47}



Fig. 4 Autoradiogram of blot hybridization analysis of *El. electricus* electroplax RNA with a cDNA probe. Total RNA (20 µg) was analysed by the procedure of Thomas²⁰. The hybridization probe used was the *Ava*II (193)–*Ava*II (1,675) fragment derived from clone pSCH411. The size markers used were calf and *E. coli* rRNA⁵⁷. Blot hybridization with other probes derived from different regions of the cloned cDNA exhibited a single hybridization-positive band with the same size; the probes used were the 577-bp fragment (excised from clone pSCH411) extending from the *Ava*II site flanking the cDNA insert to the *Ava*II (193) site, the *Ava*II (1,861)–*Hinc*II (2,422) fragment derived from clone pSCH411, the *Eco*RI/*Sal*I-excised cDNA insert of clone pSCH4 (carrying residues 2,703–4,674) and that of clone pSCH1 (carrying residues 4,716–5,141).

frame, the amino acid sequence of the cryptic portion of the sodium channel protein was deduced (Fig. 3). The translational initiation site was assigned to the methionine codon composed of nucleotide residues 1–3 because this is the first ATG triplet that appears downstream of the nonsense codon TAG (residues –42 to –40) found in-frame. This assignment is supported by the fact that the sequence surrounding the ATG triplet agrees with the favoured sequence that flanks functional initiation codons in eukaryotic mRNAs²³, that is, $\hat{A}XXAUGG$, where X can be any nucleotide; the sequences flanking the following 13 methionine codons do not match this favoured sequence. Reverse transcriptase-mediated elongation of the *Hind*III (–250)–*Hinc*II (–307) fragment derived from the 5'-terminal region of clone pSCH411 yielded a cDNA transcript of ~600 nucleotides, suggesting that no more than ~500 nucleotides are missing from the cDNA we have cloned. This is consistent with the size of the mRNA coding for the sodium channel protein (~8,000 nucleotides) as estimated by blot hybridization analysis²⁰ of electroplax RNA (Fig. 4). The codon specifying the valine residue at position 1,820 is followed by the translational termination codon TAA. The length of the 3'-non-coding region of the cDNA is 1,386 nucleotides (clone pSCH14) or 1,382 nucleotides (clone pSCH50), excluding the poly(dA) tract. Two overlapping copies of the polyadenylation signal²⁴ AATAAA (residues 6,831–6,836 and 6,827–6,832) are found 16 nucleotides upstream from the poly(dA) tract of the respective clones.

From the cDNA sequence, we conclude that the *El. electricus* sodium channel protein is composed of 1,820 amino acid residues. The molecular weight of the protein is calculated to be 208,321. This value agrees with the reported molecular weight of the protein moiety of the sodium channel protein, whose carbohydrate content is ~29% by weight⁷. The amino acid composition of the sodium channel protein predicted from the cDNA sequence compares reasonably well with that determined⁷. There are 10 potential sites of N-glycosylation²⁵, that is, Asn-X-Ser/Thr, where X can be any amino acid except proline²⁶ (asparagine residues at positions 205, 278, 288, 317, 591, 690, 797, 1,160, 1,174 and 1,806); six of them are assigned to the extracellular side of the membrane (see below).

The protein structure

Homology matrix comparison²⁷ of the amino acid sequence of the sodium channel protein reveals the presence of four internal repeats that exhibit sequence homology. Figure 5 shows the alignment of the amino acid sequences of the four repeats, which consist of residues 111–419 including a non-homologous seg-

ment of residues 285–342 (repeat I), residues 555–807 (repeat II), residues 989–1,281 including a non-homologous segment of residues 1,172–1,194 (repeat III) and residues 1,311–1,587 including a non-homologous segment of residues 1,490–1,505 (repeat IV). Of the positions aligned, 54%, 54%, 43%, 50%, 54% and 47% are occupied by identical residues or residues considered to be favoured substitutions for the I/II, I/III, I/IV, II/III, II/IV and III/IV pairs, respectively; the non-homologous segments have not been included in the calculation, and gaps have been counted as one substitution regardless of their length. Statistical tests²⁷ show that the sequence similarity among the four repeats is highly significant, except for the I/IV pair, the probability of occurrence by chance being less than 10^{-9} for the I/II, I/III, II/III and II/IV pairs and 1.3×10^{-3} for the III/IV pair. This observation strongly suggests that the four repeated homology units of the sodium channel protein have evolved from a single common ancestor by internal duplications.

The amino acid sequence of the sodium channel protein was analysed for predicted secondary structure²⁸ and for local hydrophathy²⁹ (Fig. 6A). The segments that can form structures of α -helix or β -sheet extend over a wide range of the protein molecule. The hydrophathy profile along the polypeptide chain indicates that each repetitive homology unit contains five hydrophobic segments (S1, S2, S3, S5 and S6) at equivalent positions. In addition, each unit contains a characteristic segment with strong positive charge (S4) located between segments S3 and S5.

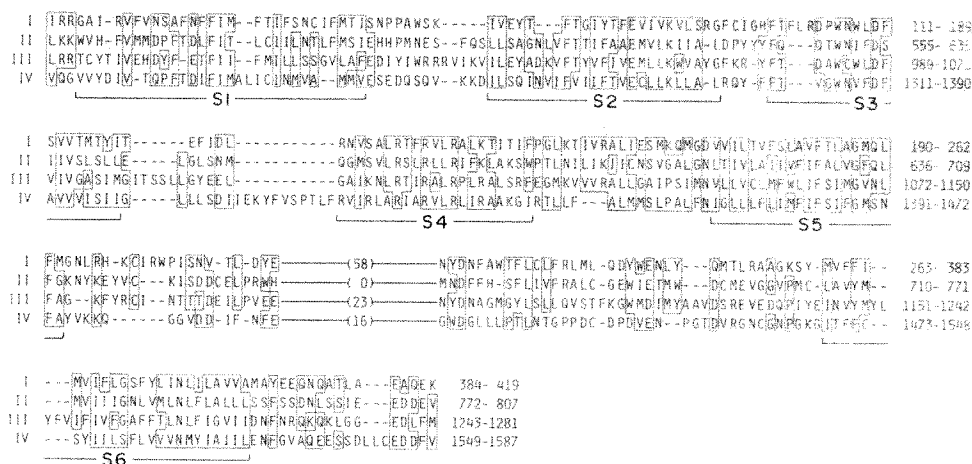
Segments S5 and S6 in each repeat correspond to highly hydrophobic regions with predicted secondary structure that comprise a continuous stretch of 24–38 uncharged amino acid residues including many nonpolar residues. These hydrophobic regions are flanked on both sides by charged residues. Because such a sequence is characteristic of transmembrane protein segments, segments S5 and S6 most probably traverse the membrane, forming α -helical structures^{30–32}.

Segments S1, S2 and S3 in each repeat represent hydrophobic regions with predicted secondary structure consisting of 18–28 amino acid residues that are largely nonpolar but include a few charged residues. Negatively charged residues predominate in segments S1 and S3, whereas both positively charged and negatively charged residues are present in segment S2, so that this segment generally has no net charge. If an α -helical structure is assumed for these segments, the charged side chains in them are clustered largely on one side of the helix, the opposite side being occupied mostly by nonpolar side chains. Thus, segments S1, S2 and S3 can also span the membrane, forming amphipathic α -helical structures.

Segment S4 in each repeat represents a positively charged region with predicted secondary structure comprising 21 amino acid residues including five to seven arginine or lysine residues; arginine residues predominate. This segment exhibits a unique structural feature in that the arginine or lysine residues are located at every third position. The residues intervening between these basic residues are mostly nonpolar. If an α -helical structure is assumed for this segment, the positively charged side chains would be distributed along a spiral line, making a three-quarter to one turn. Alternatively, if a 3_{10} -helical structure is assumed, they would lie on one side of the helix so that the segment would be strongly amphipathic. On the other hand, if a β -sheet structure is assumed, they would extend alternately towards both sides of the peptide backbone.

It seems reasonable to postulate that the four repeated homology units are oriented in a pseudosymmetric fashion across the membrane. If this is the case, each unit should contain an even number of transmembrane segments because no additional hydrophobic segments are predicted outside the homology units (see Fig. 6A). The transmembrane segments are most likely to be involved directly or indirectly in the formation of the ionic channel. As the minimum cross-section of the channel is considered to be $3 \times 5 \text{ \AA}$ (refs 33, 34), at least one helical segment from each repetitive unit would take part in constructing the inner wall of the ionic channel at the narrowest point. The remaining helical segments may surround those forming the

Fig. 5 Alignment of the amino acid sequences of the four internal homology units. The sequences of repeats I (top), II (second row), III (third row) and IV (bottom) are aligned. The one-letter amino acid notation is used, and the numbers of the residues on each line are given on the right side. Identities or favoured amino acid substitutions among three or four residues at one aligned position are indicated by boxes. Favoured amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E and Q; H, R and K; M, I, L and V; F, Y and W (ref. 58). Gaps (-) have been inserted to achieve maximum homology. The non-homologous segments in repeats I, III and IV are shown by lines; the sum of residues present in each non-homologous segment is given in parentheses. Segments S1-S6 are indicated.



channel, or some of them may face the channel in its dilated portion. In view of the strong hydrophobicity and the uncharged nature of segments S5 and S6, there seems to be little doubt that they represent transmembrane segments. It is likely that two of the three segments S1, S2 and S3 also traverse the membrane. The negatively charged side chains in segment S1 and/or segment S3 may be related to the ion selectivity of the sodium channel³⁵. The presence of a continuous stretch of 31 uncharged residues in the S1 region of homologous repeat I seems to favour the view that segment S1, together with segment S2 (or S3), spans the membrane.

The sodium channel protein, in contrast to many other transmembrane proteins³⁶, does not possess an amino-terminal sequence characteristic of the signal peptide³⁷. There are a number of such precedents³⁸⁻⁴¹, and transmembrane proteins devoid of a cleavable signal peptide are considered to have their amino terminus on the cytoplasmic side of the membrane³¹, as this is in fact the case for the erythrocyte anion transport protein band III (ref. 38). On the basis of the considerations mentioned above, the transmembrane topology of the sodium channel protein molecule has tentatively been assigned as shown in Fig. 6B. The possibility that segment S3, instead of segment S2 (or S1), traverses the membrane or that all six segments S1-S6 span the membrane cannot be excluded. In view of the ion selectivity of the sodium channel³⁵, however, it seems rather unlikely that segment S4, which has strong positive charge, is involved in the channel lining.

The voltage-dependent operation of the sodium channel implies that the structure responsible for gating is charged and is able to move in the membrane field, as postulated by Hodgkin and Huxley³ and subsequently confirmed by measurement of

the gating current⁴². The gating current is outward during activation, and available evidence suggests that both the activation and inactivation gates are located near the inner membrane surface⁴³. Noteworthy in this context is the presence of the unique, positively charged segment S4 in each of the four repeated homology units (see above) as well as the presence of a large excess of negatively charged amino acid residues over positively charged residues in the region between segment S6 of homologous repeat II and segment S1 of the repeat III. There are four segments each having 5-12 clustered acidic residues (segments composed of residues 802-806, 847-857, 894-910 and 942-955) in this region, which is assigned to the cytoplasmic side of the membrane (see Fig. 6B). These acidic segments are located at regular intervals of 31-40 residues, corresponding to the four hydrophilic segments observed in the hydropathy profile (see Fig. 6A). It is attractive to hypothesize that each of the four positively charged segments, possibly in conjunction with each of the four negatively charged segments, acts as voltage sensor, thus being involved in an activation gate. The presence of four homologous structures in a single sodium channel molecule would be consistent with the sigmoid activation kinetics characteristic of this channel³. Armstrong⁴⁴ postulates the presence of four to six closed states rather than three³ and claims that the successive transitions among them are fast and are each associated with a charge movement that amounts to only half that in the transition from the last closed state to the open state. Of interest in this context is the finding that the charge carried by the four individual acidic segments varies about twofold. Concerted movement of many negative groups and/or many positive groups as clusters even by a small distance could account for the gating current⁴⁴ and would induce a

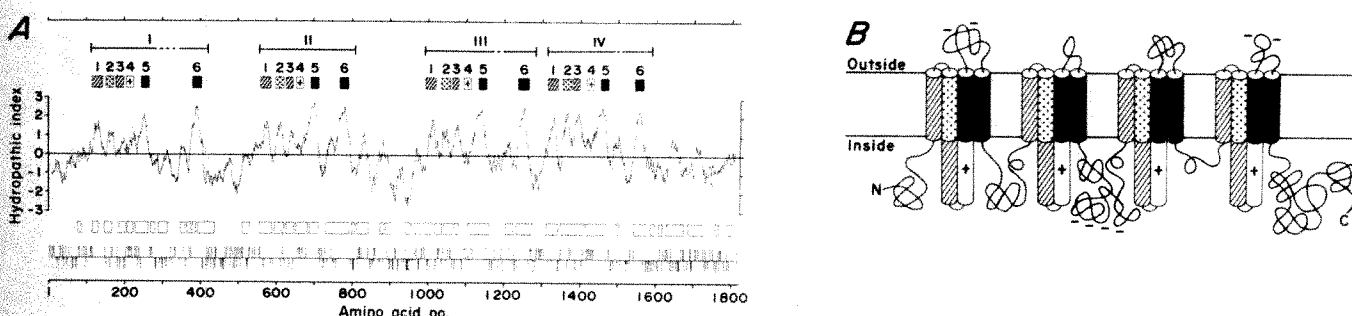


Fig. 6 Hydropathy profile and predicted secondary structures (A) and proposed transmembrane topology (B) of the sodium channel protein. In A, the averaged hydropathic index of a nonadecapeptide composed of amino acid residues $i-9$ to $i+9$ has been plotted against i , where i represents amino acid number; the hydropathy indices of individual amino acids have been taken from ref. 29. The positions of the predicted structures of α -helix and/or β -sheet²⁸ that have a length of 10 or more residues are shown by open boxes. The positions of positively charged residues (lysine and arginine) and negatively charged residues (aspartic acid and glutamic acid) are indicated by upward and downward vertical lines, respectively. The locations of homologous repeats I, II, III and IV are shown by bars, and those of the non-homologous segments within the repeats by dashed lines. The positions of the characteristic segments S1-S6 in each repeat are indicated as follows: hatched boxes, segments S1 and S3 which generally contain negatively charged residues; stippled boxes, segment S2 with both positively charged and negatively charged residues; boxes with a plus sign, segment S4 with regularly spaced, positively charged residues; closed boxes, segments S5 and S6 without charged residues. In B, the four homology units spanning the membrane, which presumably surround the ionic channel, are displayed linearly. Segments S1-S6 in each unit are shown by cylinders marked as in A, and segments S1, S2, S5 and S6 are located within the membrane. Alternative possibilities of the transmembrane topology are discussed in the text. The regions containing clustered negatively charged residues are indicated by minus signs.

conformational change of the protein to open the channel. It is also intriguing to speculate that the region encompassing the negatively charged segments, after such a conformational change upon depolarization, might in turn be involved in the inactivation of the channel, under the assumption that the activation and inactivation mechanisms are coupled⁴⁴.

The region between segments S5 and S6 of homologous repeat I as well as IV is also rich in acidic residues, which are not as clustered as those in the negatively charged region discussed above. As the region between segments S5 and S6 is assigned to the extracellular side of the membrane, it is tempting to

hypothesize that the negatively charged residues in this region of homologous repeats I and IV, facing the outer end of the channel, are also involved in the ion selectivity of the channel and that these areas contain the binding sites for basic neurotoxins⁴.

We thank Dr Paul Berg and Dr Hiroto Okayama for providing us with their high-efficiency cloning system. This investigation was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, the Mitsubishi Foundation and the Japanese Foundation of Metabolism and Diseases.

Received 20 August; accepted 18 September 1984.

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The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein

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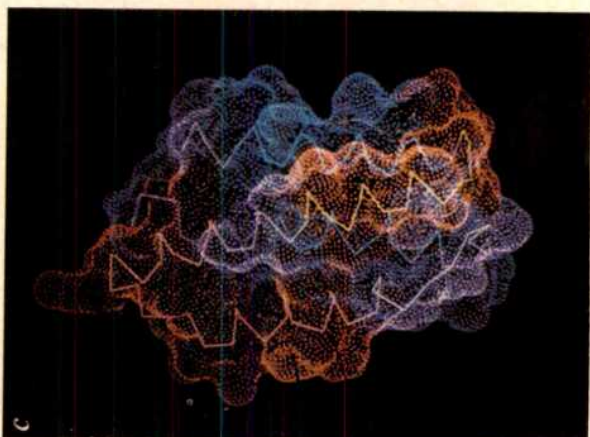
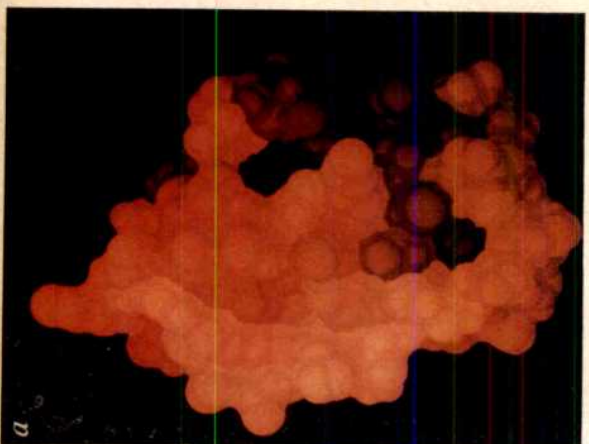
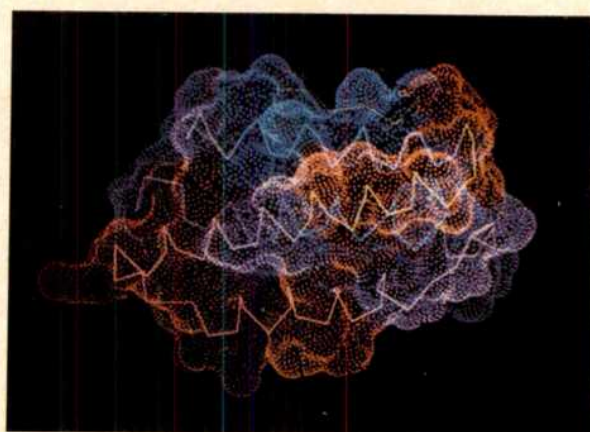
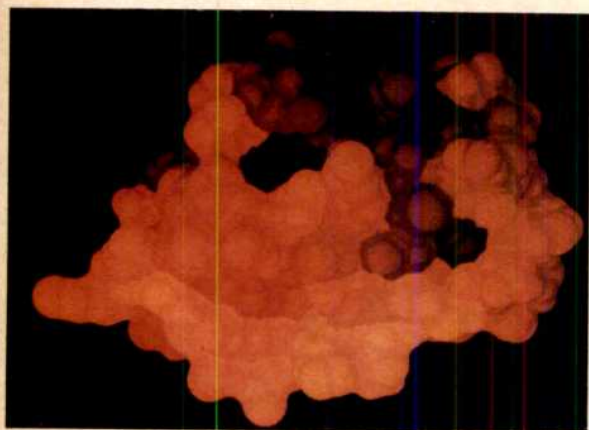
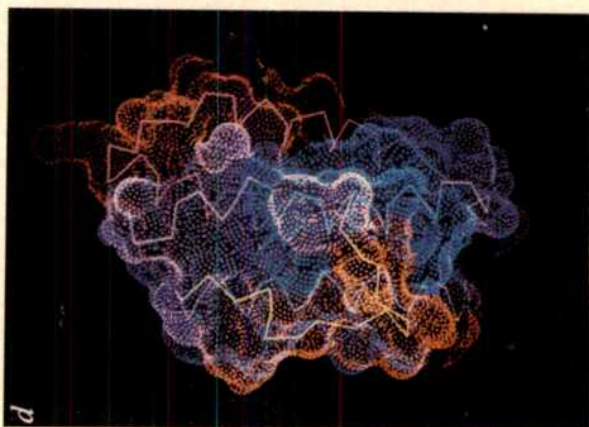
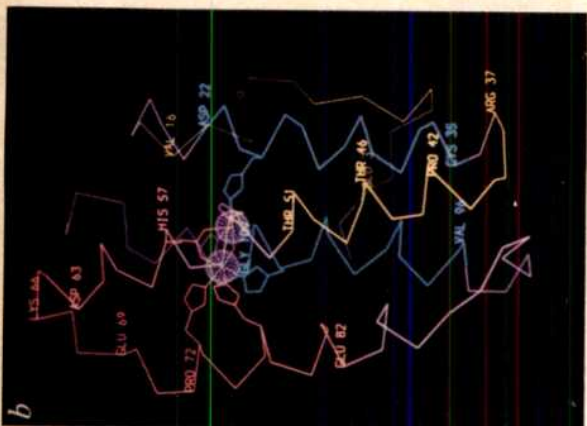
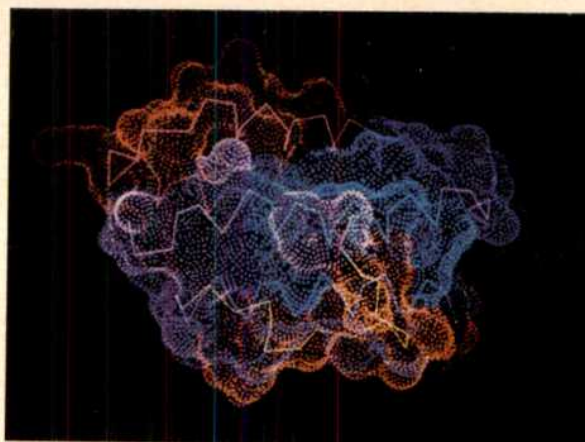
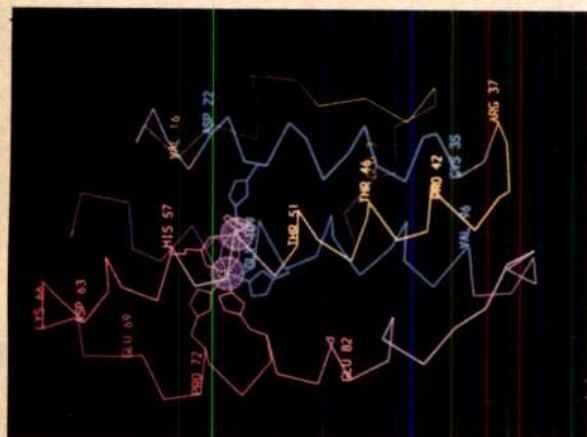
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To study the nature of antigenic recognition, antibodies have been prepared against a set of peptide sequences representing both highly mobile and well-ordered regions of myohaemerythrin, based on X-ray crystallographic temperature factors. Anti-peptide antibodies against highly mobile regions react strongly with the native protein; anti-peptide antibodies from well-ordered regions do not. Mobility is a major factor in the recognition of the native protein by anti-peptide antibodies; this may be of general significance in protein-protein interactions.

ANTIBODIES against short chemically synthesized peptides are of great use in detecting proteins and studying their structure¹. The high frequency with which these antibodies react with intact folded proteins has raised several interesting theoretical issues; one of these is addressed by considering the differences between antibodies made against intact proteins and anti-peptide antibodies. The preparation of anti-protein antibodies involves the

injection of one of the most conformationally restricted states of the molecule, the folded protein; the resultant antibodies are tested for reactivity against proteins or peptides in a state of equal or less order. The binding of these antibodies is therefore sensitive to perturbations of protein structure such as fragmentation or denaturation². Conversely anti-peptide antibodies are made against a conformationally less restricted protein site and



then tested against a protein molecule which is almost always more ordered.

To explain why antibodies to short, flexible peptides frequently react with more ordered molecules, we have considered models involving local disorder in proteins^{3,4}. We envision that sites in proteins move through a range of conformations and bind to anti-peptide antibodies only when a conformation is displayed which the antibody recognizes. Thus the antibodies act as a 'sink', trapping particular local conformations in mobile areas of proteins. Here we test this model's prediction that mobile sites in proteins will be recognized preferentially by anti-peptide antibodies.

Differential mobility

Refined high resolution X-ray crystal structures of molecules identify atomic positions and provide empirically determined information about atomic mobility in the form of the atomic temperature factors defined by $8\pi^2 u^2$, where the mean square displacement u^2 is averaged over time and the crystal lattice⁵. Patterns of temperature factors can reveal the lower-frequency concerted motions of groups of atoms, indicating the relative conformational variability of different regions^{6,7}; high factors indicate shallow potential wells (low energy barriers between

different conformations) allowing access to multiple conformations at biological temperatures. Individual atomic temperature factors are named for the temperature dependence of the velocity of atomic motion and the corresponding thermal energy available for surmounting these conformational potential energy barriers, rather than for local temperature differences within the molecule. (For simplicity, we use the suggestive terms 'hot' and 'cold' to refer to highly mobile (high temperature factors) and well-ordered regions (low factors), respectively.) Temperature factors may also reflect static disorder in the crystal, error in the absorption corrections, improper scaling and errors in the interpretation of the electron density. By examining the overall pattern of atomic temperature factors for a highly refined protein structure which has been solved independently in different crystal systems, the differential mobility of various regions of the molecule can be isolated from these other factors⁸.

To characterize patterns of temperature factors on protein surfaces, we examined 22 X-ray structures available from the Brookhaven Protein Data Bank⁹. We mapped temperature factors onto the solvent-accessible molecular surfaces¹⁰ and found them to be characterized by areas of both high and low mobility. Regions of low mobility were often, but not always, associated with interfaces between subunits in multimeric proteins or between adjacent molecules in the crystal lattice; such contacts were characterized by a lower than average ratio of side-chain to main-chain temperature factors. Otherwise, the average main-chain and atomic temperature factors also reflected the expected relative mobility of different parts of the molecular surface based on the constraints from underlying stereochemical interactions.

The differential mobilities of protein surfaces suggested to us that crystallographic temperature factors might be relevant in antibody-antigen recognition, and that this could be examined experimentally with anti-peptide antibodies. To select a protein for this study, we compiled a database of crystal structures known at sufficient resolution to provide accurate parameters of thermal mobility. We then eliminated proteins with considerable sequence homology to rabbit proteins (thus avoiding immunological tolerance) and proteases (which could complicate the immunoassays). We chose myohaemerythrin from the remaining protein structures for its well-refined temperature factors, wide range of mobility at the molecular surface and contiguous regions of the amino acid sequence characterized by either high or low mobility.

Myohaemerythrin belongs to the haemerythrin family of oxygen-carrying proteins found in some invertebrates. The structural fold for this family of proteins is an antiparallel bundle of four helices¹¹ (A, B, C and D) surrounding a two-iron centre at the active site, with a loop region at the N-terminus and shorter loops between the helices and at the C-terminus. Three-dimensional crystal structures with temperature factors have been refined at high resolution for monomeric myohaemerythrin from the marine worm *Themiste zostericola* (S. Sheriff, W.A.H. and J. L. Smith, unpublished results) and octameric haemerythrin from *Themiste dyscrita*¹². The structure of myohaemerythrin is known to 1.7 Å resolution in two crystalline directions (*a* and *c*) and to 1.3 Å in the third direction (*b*), with a crystallographic residual error of 0.159 and a r.m.s. deviation from ideal bond lengths of 0.017 Å. The related structure of haemerythrin is known to a resolution of 2.0 Å, with a residual error of 0.175 and a r.m.s. deviation from ideal bond lengths of 0.026 Å. The temperature factors for these two crystal structures are unique in being empirically corrected for packing contacts⁴⁵. Myohaemerythrin (Fig. 1a) demonstrates variable surface mobility of different intra-domain structural elements typical of high resolution protein structures. Before adjustment for the effects of crystal contacts, the A and D helices are relatively highly ordered, the C helix is highly mobile and the B helix varies from being relatively well ordered at the N-terminal end to relatively mobile at the C-terminal end. The 'glowing coal' temperature scale (Fig. 1a) delineates clearly this partitioning of the exposed surface of myohaemerythrin into regions of high and low mobility.

← **Fig. 1** Stereo pairs showing the α -carbon backbone and the molecular surface of myohaemerythrin colour-coded by temperature factor. The molecular surface calculation^{25,32} rolls mathematically a water-sized probe sphere (1.4 Å radius) representing a solvent water molecule over the van der Waals' surface of the protein. All molecular surface calculations used individual van der Waals' radii that included implicit hydrogen atoms^{33,34}. Using the general purpose graphics modelling language GRAMPS³⁵ and the molecular modelling program GRANNY³⁶, the protein skeletal model together with its associated molecular surface (shown as a transparent set of colour-coded dots) was displayed interactively using vector computer graphics. In these figures, the terms front and back refer to the stereo directions towards and away from the reader, respectively, while all other directional terms refer to locations within the plane of the page. **a**, Patterns of mobility on the molecular surface of myohaemerythrin. This stereo view shows the solid external molecular surface colour-coded by the average main-chain temperature factors using a radiating-body colour scale. The molecule is oriented with the helices vertical, and the two-iron centre towards the top (*b* shows the skeletal model in the same orientation). The darkest colours (lowest temperature factors) are associated with the A helix (lower right) and the brightest colours (highest temperature factors) with the C helix (upper left). In these temperature factors, before adjustment for the effect of crystal contacts, the B helix (centre) varies from low (bottom, centre) to high (top, centre) temperature factors. The low temperature factors in the B helix apparently result from crystal packing contacts. This raster colour graphics representation was calculated using RAMS³⁷ and displayed on an AED raster graphics display. **b**, The synthetic peptide positions shown on the myohaemerythrin α -carbon backbone. Peptides are coloured to identify hot (red), cold (blue) and hot/contact (yellow) types of mobility, (magenta, not studied). The iron ions are shown by magenta spheres and bonds. The side chains of the iron liganding residues (His 25, His 54, Glu 58, His 73, His 77, His 106 and Asp 111) are colour-coded to match the peptide sequences in which they occur. Residues at the end of each non-nested peptide are labelled at their respective α -carbons. Hot peptides 57-66, 63-72, 69-82, 73-82 appear upper left, cold peptides 22-35, 26-35 right, 96-109, 100-109 at the centre (back), hot/contact peptides 3-16, 7-16 at the right (back) and 37-46, 42-51 at the centre (front). **c**, The location and exposed molecular surface of peptide sequences shown in the same orientation as **a** and **b**. The molecular surface and α -carbon backbone of the peptides are colour-coded to identify four categories of peptides: highly mobile in the crystallographic temperature factors (red backbone, molecular surface), highly mobile in the temperature factors adjusted for crystal contacts (yellow backbone, red molecular surface), relatively well ordered (blue backbone, molecular surface) and peptides not studied (magenta backbone, molecular surface). The peptides studied all show large amounts of exposed surface area. **d**, The location and exposed molecular surface of peptide sequences as described for **c**, but shown in the opposite orientation.

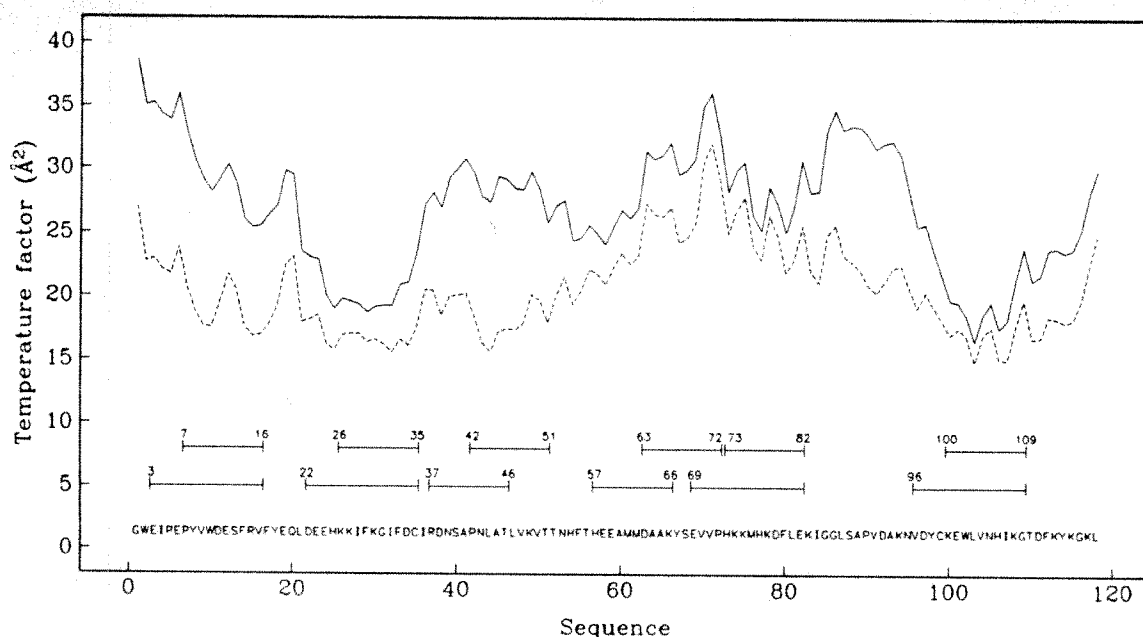


Fig. 2 Plot adapted from Sheriff *et al.*⁴⁵ of the average main-chain temperature factors along the polypeptide chain before (dashed line) and after (solid line) adjustment for crystal contacts. Regions 1–17 (N-terminal loop), 38–50 (N-terminus of the B helix), and 85–94 (C–D loop) show the greatest increase in relative mobility when corrected for crystal contacts. The 118 residue amino acid sequence of myohaemerythrin³⁸, corrected by a sequence inversion of residues 34 and 35 revealed by X-ray crystallographic studies (J. L. Smith and W.A.H., unpublished results), is shown in one-letter code (A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine). 10 and 14 residue sequences of the protein chosen for peptide synthesis are indicated by sequence-numbered bars. To allow coupling to the immunological carrier protein, an additional cysteine residue not found in the primary sequence was synthesized at the C-terminus of each peptide. Additionally, cysteine 35 in peptides 22–35 and 26–35 was replaced with a serine to avoid multiple coupling to the carrier, while maintaining peptides of a fixed length.

Methods: Peptides were synthesized as described previously³⁹ by the solid phase method^{40,41} using a Beckman model 990B peptide synthesizer. The initial amino acid resin was prepared by esterification of Boc-(MeoBzl)-Cys-OH (2.0 g anhydrous-caesium salt, 4.3 mequiv.) to chloromethylated resin (polystyrene/1% divinylbenzene, bio-beads S.X.-1, 200–400 mesh, 0.70 mmol g⁻¹) in 25 ml anhydrous dimethylformamide (DMF) by stirring for 24 h at 50 °C. A threefold excess of caesium salt over active chloride polymer gives almost complete substitution. Substitution was determined to be 0.55 mequiv. g⁻¹ by the picric acid test and amino acid analysis, and 0.53 mequiv. g⁻¹ following HCl-propionic acid hydrolysis. For each synthesis, 1.0 g (0.53 mequiv.) of Boc-(MeoBzl)-Cys resin was used. The following side-chain protecting groups were used: *O*-Br-Z for lysine and tyrosine; *O*-benzyl for threonine, serine, aspartic acid and glutamic acid; tosyl for arginine; *N*-formyl for tryptophan; *N*-dinitrophenyl for histidine. Protected amino acids (Vega Biochemicals or Peninsula Laboratories) were recrystallized from the appropriate solvent to give single spots on TLC (chloroform/acetic acid = 15:1 or chloroform/methanol = 10:1). All couplings were carried out using a 10-fold excess of Boc-AA-OH. For asparagine and glutamine, an equimolar amount of *N*-hydroxybenzotriazole was added to the protected amino acid and DMF was used as the solvent. All coupling reactions were ≥99% complete by the picric acid test. The removal of dinitrophenyl from histidine-containing peptides was accomplished with *N*-methyl mercaptoacetamide⁴² in DMF by mixing with the resin-bound peptide for 15 min. Removal was >95%. The resin bound protected peptides (500 mg to 2.5 g, 0.12–0.60 mequiv.) were treated with twice their weight of anisole and with 40 times their volume (relative to weight) of anhydrous HF at 4 °C for 1 h. For peptides containing glutamic acid, 5% v/v pyridine was added to the HF to prevent anisylation. After evaporating the HF with a stream of N₂, the red/yellow residue was mixed with anhydrous ether (50 × w/v) and stirred vigorously for 15 min; after repeating the ether extraction three times, the white residue was filtered and dried *in vacuo*. This dried resin-peptide mixture was extracted with 5% HOAc, lyophilized and amino acid analysis performed (~90% removal of the peptide from the resin). For peptides containing tryptophan, the *N*-formyl protecting group was removed from the peptide by treatment for 1.5 min at pH 11.5 (NaOH) in the presence of hydrazine. The amino acid analysis was within 5% of the theoretical value for all peptides. All peptides were coupled²⁷ to the immunogenic carrier protein KLH through the C-terminal cysteine of the peptide using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as the coupling reagent⁴³. Two rabbits were immunized with each peptide in a course of 3 injections of 200 µg peptide-coupled KLH (in complete Freund's adjuvant (1:1) subcutaneously on day 0, in incomplete Freund's adjuvant (1:1) subcutaneously on day 14, and with 4 mg of alum intraperitoneally on day 21).²⁷ Rabbits were bled 10 days after the third injection and boosted 3 months from the last injection and bled again 7 and 14 days later.

Myohaemerythrin peptides

Peptides, representing both helical and non-helical regions of myohaemerythrin, were selected on the basis of main-chain temperature factors averaged by residue, because the main-chain mobility reflects the global conformational variability of the protein (Fig. 1b). Three of the hot peptides include residues liganding the irons; in the native protein the mobility of these sequences is limited to conformations that do not disrupt the metal-ligand geometry.

Figure 1b–d shows the locations and exposed surfaces of the synthesized peptides in the protein. Of the exposed molecular surface area of myohaemerythrin (5,846 Å²), our synthesized peptides account for 4,005 Å² or ~69%. The four peptides synthesized for each of three categories encompass roughly

equal parts of the protein surface area: 23.0% hot, 19.3% cold and 26.2% hot/contact (hot after correction for crystal contacts), and all peptides have large areas of exposed molecular surface available for interaction with antibodies (Fig. 1c, d). Highly exposed amino acid residues tend to be less hydrophobic and, due to their external, relatively unconstrained position, more highly mobile than internal residues^{13,14}. We chose peptides to minimize differences in average exposed area and hydrophobicity per residue. Although there is some correlation of exposed area and mobility among the selected peptides, this is much stronger for side-chain than for main-chain atoms (Table 1). In fact, peptides with very similar average exposed areas may either be cold (peptides 22–35, 26–35 and 96–109) or hot (7–16, 42–51 and 57–66). There is very little correlation between temperature factor and average hydrophobicity per residue for the peptides chosen (Table 1).

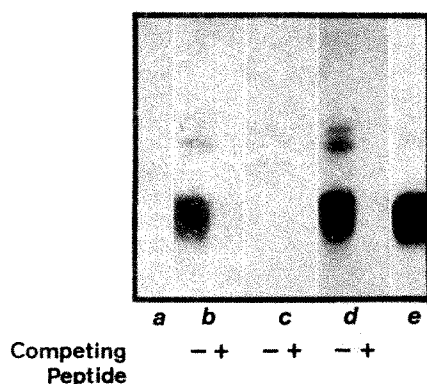


Fig. 3 Comparison of the anti-protein reactivities of antibodies raised against hot, cold, and hot/contact peptides. Lanes are representative polyacrylamide gel electrophoresis of the immunoprecipitates in the presence (+) and absence (-) of competing peptide. Lane *a*, control normal rabbit serum; lane *b*, hot peptide 57-66; lane *c*, cold peptide 26-35; lane *d*, hot/contact peptide 3-16; lane *e*, intact myohaemerythrin. Radiolabelled pellets from the immunoprecipitation were dissolved in 50 μ l of sample buffer⁴⁴ containing 2% SDS, 5% 2-mercaptoethanol and 0.001% bromophenol blue, boiled for 3 min, centrifuged to remove *S. aureus*, subjected to electrophoresis on 12.5% SDS gels, and autoradiographed. Faint bands representing aggregated states of myohaemerythrin can also be seen.

The sequence location and identity of the synthesized peptides is overlaid on the plot of the average main-chain temperature factors in Fig. 2. The 12 peptides synthesized include 83 of the total 118 residues or ~70% of the myohaemerythrin sequence. The clear division of the peptides into highly mobile and well-ordered categories is apparent in the adjusted temperature factors: two of the helices are relatively highly ordered, whereas the remainder of the structure shows significantly more mobility.

Antibody-reactive regions

To elucidate the role of molecular surface mobility in the recognition of the native protein, we raised polyclonal antibodies against synthetic peptides with each selected sequence (Table 2). Polyclonal antibodies enabled us to sample the entire range of allowed conformations for the injected synthetic peptide, whereas monoclonal antibodies would select single, possibly atypical conformations of each peptide.

The anti-peptide antisera were originally assayed for reactivity against the homologous immunizing peptide by enzyme-linked immunoabsorbent assay (ELISA). All but one of the synthetic peptides are immunogenic (Table 2); their relative immunogenicity is not correlated with their mobility. We next assayed the anti-peptide antisera for reactivity with myohaemerythrin (Table 2). All antisera raised against cold peptides (22-35, 26-35, 96-109 and 100-109) gave negative or low reactivity with the protein, whereas all antisera raised against hot peptides (excluding peptide 42-51, which did not raise any antibodies) gave higher reactivity against the protein. Of the antisera against hot peptides, those with the lowest myohaemerythrin reactivity (57-66 and 63-72) also had the lowest reactivity against their respective homologous immunizing peptides.

We used the method of Farr¹⁵ to estimate the avidity or effect of dilution on the binding of anti-peptide antisera to myohaemerythrin at five concentrations (1, 5, 10, 50 and 100 pmoles). The relative averaged avidity of the anti-peptide antisera against the protein (peptides 3-16 > 7-16 > 37-46 > 57-66 > 63-72, 69-82, 73-82, 96-109) matched the pattern of relative reactivity, antisera with higher titres generally showing greater avidity. (The negative or low reactivity of the remaining anti-peptide antisera with the protein precluded measurement of their avidities.) ELISA analyses may involve some denaturation of the target protein. We therefore assayed immunoprecipitation in conditions preserving the native structure of myohaemerythrin. Retention of the characteristic visible spectrum with a 338 nm peak¹⁶ (due to the iron centre) verified that the ligand environment remains unchanged. The results of these immunoprecipitation studies and the associated gel electrophoresis analysis (Table 2, Fig. 3) are consistent with the ELISA analyses.

To confirm the specificity of the anti-peptide antisera, we also performed immunoprecipitation studies in the presence of homologous and heterologous peptides (Table 2, Fig. 3). The anti-myohaemerythrin reactivity of antisera raised against the hot peptides was strongly inhibited by incubation with the corresponding homologous peptide, whereas incubation with the irrelevant heterologous peptide (synthesized from a sequence for influenza virus haemagglutinin) did not inhibit anti-myohaemerythrin reactivity, thus confirming the specificity of the antisera. Interestingly, antisera against cold peptides that showed low reactivity with the protein (22-35, 96-109) were, at most, only slightly inhibited by incubation with the corresponding homologous peptides. This suggests that the anti-myohaemerythrin reactivity of antibodies raised against these

Table 1 Myohaemerythrin peptide data

Sequence position	Secondary structure	Type	Mobility			Average exposed area	Average hydrophobicity
			Main*	Main	Side*		
3-16	N-terminal	Hot*	30.4	19.9	36.2	52.3	0.02
7-16	N-terminal	Hot*	28.6	18.8	32.4	43.8	0.02
22-35	A-helix	Cold	20.4	16.8	26.2	40.6	-0.16
26-35	A helix	Cold	20.1	16.6	26.2	43.8	0.05
37-46	A loop	Hot*	28.9	18.5	30.7	59.7	-0.23
42-51	B helix	Hot*	28.4	17.8	31.8	44.9	0.26
57-66	B helix	Hot	28.0	24.2	30.2	45.6	-0.11
63-72	B-C loop	Hot	31.9	27.4	34.1	60.0	0.05
69-82	C helix	Hot	29.6	26.2	35.9	54.9	-0.18
73-82	C helix	Hot	27.9	24.9	35.2	55.4	-0.41
96-109	D helix	Cold	20.8	17.6	26.7	40.1	0.04
100-109	D helix	Cold	19.4	16.9	25.1	36.7	-0.01

Peptides are numbered by their respective sequence position in myohaemerythrin. All mobilities are derived from the temperature factor data for the intact protein⁴⁵. Peptides are classified into three types of mobility: hot (high average main-chain temperature factors), cold (low average main-chain temperature factors*) and hot* (hot/contact, high average main-chain temperature factors after correction for the effects of crystal contacts). Mobility in $\text{\AA}^2$ is expressed as average main chain* (calculated from the atomic temperature factors for N, C α , C, O atoms adjusted for crystal contacts), average main chain (calculated from the atomic temperature factors for N, C α , C, O atoms), and average side chain* (calculated from the atomic temperature factors for side-chain atoms adjusted for crystal contacts). Average exposed area in $\text{\AA}^2$ is calculated using the molecular surface program MS²⁵. Average hydrophobicity is calculated using the Eisenberg consensus scale²⁶. Properties were first averaged for each residue, then these residue averages were averaged for each peptide.

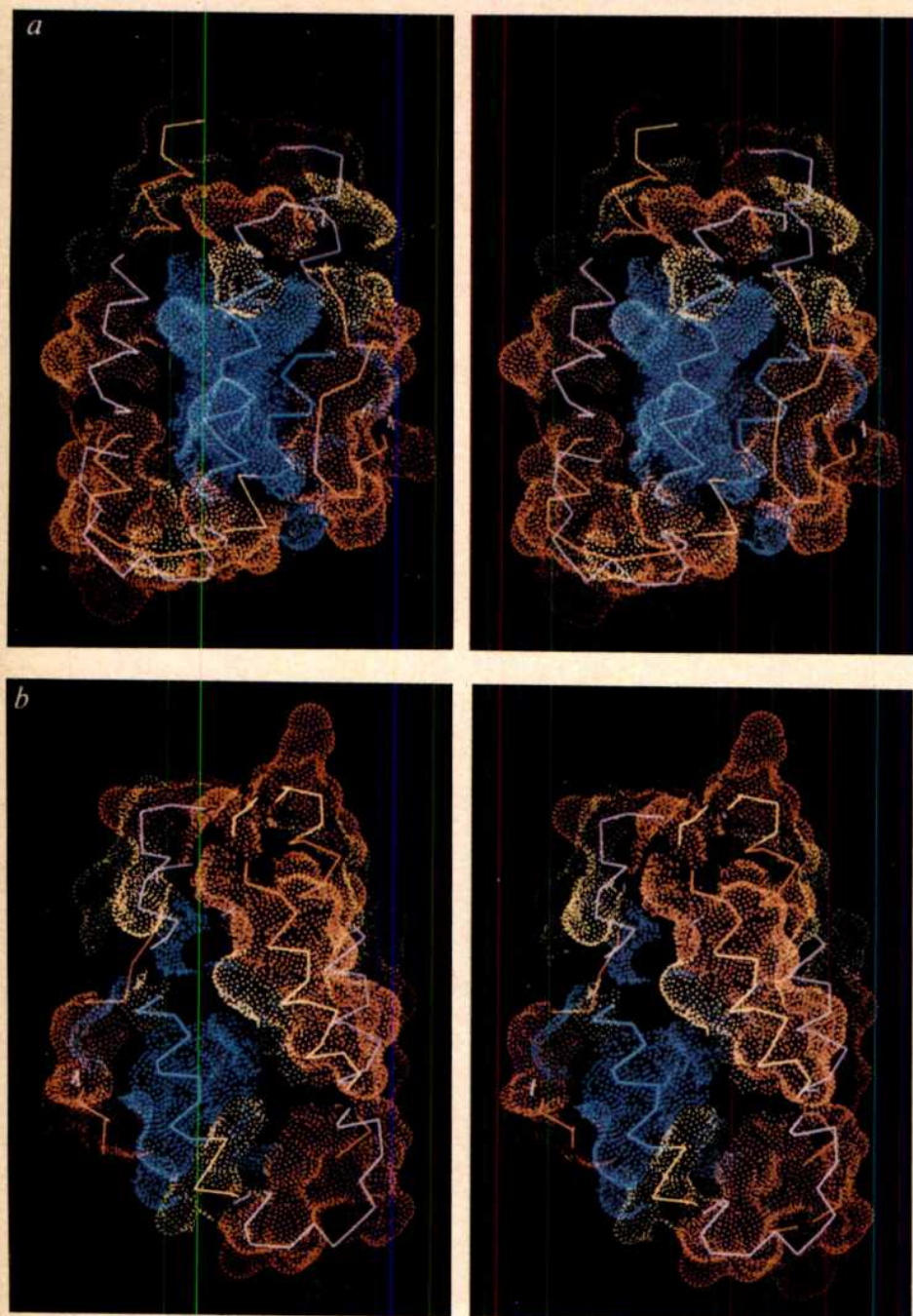


Fig. 4 Stereo views showing the correlation between the mobility of sites in a protein and their reactivity with anti-peptide antibodies. The α -carbon backbone is colour-coded by antigenic reactivity based on the immunoprecipitation results (Table 2): red, high ($> 3,500 \text{ c.p.m.}$), yellow, medium ($2,000-3,000 \text{ c.p.m.}$), blue, low ($< 200 \text{ c.p.m.}$), magenta, not studied. The exposed molecular surface is colour-coded by average adjusted main-chain temperature factors (Table 1): red, hot ($\geq 27 \text{ \AA}^2$), yellow, medium ($22-27 \text{ \AA}^2$), blue, cold ($< 22 \text{ \AA}^2$). To identify the portions of the molecular surface associated with each synthesized peptide sequence, the myohaemerythrin molecule is dissected into individual peptides (backbone and associated molecular surface) shown in a slightly exploded view. Since the peptides were coupled onto the KLH carrier at the C-terminal amino acid, overlapping and nested peptide sequences were assigned the level of reactivity associated with the sequence of the N-terminal portion of the relevant peptide. *a*, The following peptide regions (correspondence of secondary structure descriptions to sequence positions defined in Table 1) show the correlation of their temperature factors with antigenic reactivity: the N-terminus (red backbone and surface, lower right), the A helix (blue backbone and surface, centre), the A loop (red backbone and surface, lower left, front) and the C-terminal end of the B helix (red and yellow backbone and surface, upper left). *b*, The relationship is apparent for the remaining peptide regions: the non-overlapping fragment of the B-C loop (yellow backbone and red surface, top), the C helix (red and yellow backbone and surface, upper right), and the D helix (blue and yellow backbone and surface, lower left).

cold peptides may largely represent either nonspecific binding or low avidity.

The striking correlation seen in both types of immunoassay between the mobility of sites in a protein and their reactivity with anti-peptide antibodies is seen when the reactivities of the anti-peptide antibodies are mapped onto the α -carbon backbone and the average adjusted main-chain temperature factors mapped onto the external molecular surface (Fig. 4). Of the cold peptides, only antibodies to peptide 96-109 have reactivity with intact myohaemerythrin; this peptide has four residues with higher temperature factors on its N-terminal end. Of the hot peptides, the N-terminal loop peptide 3-16 represents the target site with the highest mobility and reactivity with anti-peptide antibodies. Reduced mobility due to structural constraints in myohaemerythrin resulting from the metal liganding may modulate the degree of antigenic reactivity. In the immunoprecipitation assays, antisera against the shorter of

the two nested peptides containing two metal ligands in the intact molecule (73-82) have the lowest anti-protein reactivity of any hot peptide antisera. These results support the correlation between mobility and reactivity with anti-peptide antibodies.

Part of the effect seen in these experiments could be the result of differences in the level of antibody raised against the individual peptides. Our basic findings remained the same when reactivities were normalized as a function of the anti-peptide titres: thus expressed, the anti-protein reactivities improved for antisera against hot peptides and changed little for those against cold peptides. Furthermore, we found that the increase in anti-peptide titres after the rabbits received booster injections of peptides led to increases in anti-myohaemerythrin reactivity for the antibodies raised against the hot peptides, compared with little or no increase in reactivity for the antibodies raised against the cold peptides (data not shown).

Conclusions

The ability of anti-peptide antibodies to recognize their corresponding intact folded proteins is best understood as a dynamic process which is most favoured when the conformation of the peptide immunogen approximates the target site in the protein and that site is mobile. The relative contributions of each factor to the success of an immunogen are difficult to assess and may vary from peptide to peptide. Our data suggest that recognition involves the interaction of antibodies with sites in the protein which can adopt multiple conformations that may differ from the average structure seen by X-ray crystallography. One may envision a process in which the initial interaction with the antibody is sufficient for recognition of the appropriate site, after which considerable induction of shape may take place.

Table 2 Reactivity of anti-peptide antisera

Sequence position	ELISA titres		Immunoprecipitation	
	α -Peptide vs peptide	α -Peptide vs myohaemerythrin	^{125} I-Myohaemerythrin precipitated (c.p.m.)*	% Inhibition by peptide†
3-16	640-1,280	12,800	14,849	97.1
7-16	640-1,280	3,200	7,384	92.6
22-35	1,280-2,560	—	180	0.0
26-35	320-640	60	0	0.0
37-46	640-1,280	9,500	4,026	100.0
42-51	—	—	0	0.0
57-66	160-320	600	7,277	80.0
63-72	160-320	375	2,543	77.6
69-82	>2,560	1,050	3,774	91.3
73-82	>2,560	1,400	2,154	62.0
96-109	1,280-2,560	200	2,617	22.5
100-109	320-640	—	0	0.0

Reactivities of the various anti-peptide antisera raised in pairs of rabbits were assayed against the antigen in the solid phase using ELISAs and against the antigen in solution using immunoprecipitation. ELISA titres are expressed as the reciprocal of antibody dilution extrapolated to bind 50% of 50 pmol antigen per well. ELISA: Assays followed the procedure described by Green *et al.*²⁷ 50 pmoles antigen (synthetic peptide or myohaemerythrin molecule) in phosphate-buffered saline (PBS, 10 mM sodium phosphate, 0.15 M NaCl, pH 7.2) were added to each well of a polystyrene microtitre plate and dried overnight at 37 °C. The dried antigen was then fixed to the plate by treatment with 50 μ l of methanol per well for 5 min at room temperature. The plates were coated for 4 h with a solution of non-fat dry milk (BLOTTO, ref. 28) to block nonspecific absorption of antibodies to the plate. Following the addition of 25 μ l antiserum (serially diluted with the same solution) to each well, the plates were incubated overnight at room temperature and the unbound antibody was washed off with water. The antigen-antibody complex was reacted with goat anti-rabbit IgG coupled to glucose oxidase²⁹. After removal of excess goat anti-rabbit IgG with water, this sandwich complex was measured enzymatically by the addition of enzyme substrate. For less denaturing (wet plate) ELISAs, the antigen was attached to the plates by overnight incubation in 15 mM saline in 10 mM phosphate buffer, pH 9.0. Non-immunogenic peptide 42-51 continued to give negative results in repeated ELISA experiments. Rabbit antisera against the intact myohaemerythrin protein were tested by ELISA for reactivity with the protein and with the synthetic peptides. The anti-protein antisera showed high reactivity (titres > 1,280) against myohaemerythrin, but did not react with any of the tested synthetic peptides. Controls showed no cross-reactivity between kehole limpet haemocyanin (KLH) and myohaemerythrin and no anti-myohaemerythrin reactivity in preimmune serum. Immunoprecipitation: Myohaemerythrin was radiolabelled with ^{125} I by the chloramine-T reaction³⁰ to a final incorporation of about 25 μ Ci μ g⁻¹. After dialysis, 10⁶ c.p.m. of ^{125} I-myohaemerythrin in PBS were reacted with 10 μ l of anti-peptide antiserum for 60 min on ice. The antigen-antibody complexes were precipitated with formalin-fixed *Staphylococcus aureus*. The pellets were washed once with RIPA buffer (PBS containing 1% Nonidet P-40, 0.05% sodium deoxycholate and 0.1% SDS) and twice with 500 mM LiCl, 100 mM Tris (pH 8.5), then analysed for radioactivity. In peptide competition experiments, the anti-peptide antibodies were preincubated for 60 min with 100 μ g peptide in PBS before the addition of ^{125} I-myohaemerythrin. Western blot analysis: The correlation of mobility with antigenic reactivity was also studied by Western blot analysis³¹. Following polyacrylamide gel electrophoresis of myohaemerythrin in denaturing conditions (the samples were boiled in the presence of 2% SDS and 5% 2-mercaptoethanol), the protein was transferred to a nitrocellulose filter and incubated with selected anti-peptide antisera. Detection of the bound antisera with ^{125} I-labelled *S. aureus* protein A showed that the antisera against cold peptides did not react well with myohaemerythrin in these conditions, whereas antisera against hot peptides showed relatively strong reactivity.

* Average of two independent experiments, after correction for nonspecific binding.

† 100.0 % minus per cent of activity remaining in the presence of the peptide.

Mobility is thus involved in both adoption of protein conformations suitable for recognition by the antibody and the degree to which a given region can be induced into a conformation that fits the antibody binding site.

For proteins in solution, the range and extent of mobility are probably greater than those calculated from the average main-chain temperature factors; dynamics calculations indicate that conformational flexibility is greater than that seen in the X-ray crystal structures¹⁷. In myohaemerythrin, the highest temperature factors predict average isotropic displacements between ± 0.7 and ± 0.9 Å for the main and side chains, respectively. Rather than defining exactly the excursions of individual residues¹⁸⁻²⁰, however, these high values identify areas with relatively low energy barriers between alternate conformations. The overall mobility does not imply any complete local unfolding in myohaemerythrin, as no visible spectroscopic change in the ligand environment of the two iron atoms occurs in the solvent and salt conditions used in the present study. The fact that strong protein-reactive antibodies can be induced by peptides representing highly mobile sites in the protein that contain iron binding residues (Fig. 1b) indicates that mobility, but not complete local disorder, is important for antigenic reactivity.

The anti-protein reactivity of antibodies to synthesized myohaemerythrin peptides correlates better with the mobility of the target sites than with their exposed surface area. The cold peptides 22-35, 26-35 and 96-109 have similar average exposed surface areas to hot peptides 7-16 and 57-66, but much less reactive antisera. Thus, the apparent relationship between exposed area and antigenic reactivity²¹ may actually reflect the increased mobilities of turns, loops and other highly exposed areas. As a large percentage of the exposed molecular surface (80% in myohaemerythrin) belongs to highly mobile residues, our results are consistent with studies¹ showing that most anti-peptide antibodies react with the corresponding intact protein.

Protein-protein interaction is a complex process involving many characteristics of the molecular surface, including shape complementarity and hydrophobicity of the interacting molecular surfaces²². The mapping of electrostatic potential onto molecular surfaces^{23,24} has revealed the importance of electrostatic forces in intermolecular interactions. Our present results showing a correlation between the mobility of sites in a protein and their reactivity with anti-peptide antibodies suggest that molecular mobility is an essential part of the antigenic recognition process and perhaps of protein-protein recognition in general. The distinguishing feature of anti-peptide antibodies is their predetermined specificity. Elucidation of the amino acid sequence of a protein should allow the use of site-specific anti-peptide antibodies for the investigation of the role of conformational flexibility in protein-protein recognition processes.

The sample of myohaemerythrin was prepared by the late G. L. Klippenstein, University of New Hampshire. We thank S. Sheriff for advice and assistance in the analysis of the protein coordinates and temperature factors; Leonid Volfson, Susan Gruber, Sarah Hoffmann, Susie Morrell and Andrea Struthers for technical assistance; Michael Connelly, Ian Wilson, Michael Pique and Wilfred van Gunsteren for useful discussions. J.A.T. and E.D.G. are Damon Runyon-Walter Winchell Fellows. This work was supported in part by a NIH program-project grant.

Note added in proof: Recent studies show that existing antibodies bind regions of high mobility in tobacco mosaic virus coat protein, lysozyme and myoglobin⁴⁶ and in other known protein structures⁴⁷.

Received 5 June; accepted 30 August 1984.

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LETTERS TO NATURE

Near-infrared observation of the circumsolar dust emission during the 1983 solar eclipse

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A near-infrared excess emission superposed on the coronal continuum at $\sim 4R_{\odot}$ from the Sun was first observed at $2.2\text{ }\mu\text{m}$ by Peterson¹ and MacQueen² during the total solar eclipse in 1966, and was later confirmed by MacQueen² using a balloon-borne coronagraph. Two subsequent observations were made; one³ obtained a similar result, while the other⁴ detected no excess emission. The excess emission was thought to be due to thermal radiation by a dust cloud around the Sun, and interpreted to be interplanetary dust². Brecher *et al.*⁵, on the other hand, have proposed that the ring is composed of much larger objects (of about 10 km diameter). To obtain more definite information, we have therefore carried out observations of near-infrared brightness distributions of the solar corona, using a balloon-borne photometer at a balloon altitude, during the total eclipse on 11 June 1983 in Indonesia. As we report here, emissions in excess of the strong coronal background emission were recorded in some of the scans at $\sim 4R_{\odot}$ from the Sun. The spatial distribution of the excess emission implies the existence of a circumsolar ring of dust lying approximately in the ecliptic plane.

The balloon-borne instrument we used for near-infrared photometric scanning consisted of a 16-cm Cassegrain telescope and a simultaneous four-band photometer in a reaction wheel-stabilized gondola, together with a high-sensitivity television camera. The photometer had four 4-element PbS detector arrays thermoelectrically cooled at separated foci with three beam-splitters. The infrared bands investigated were $1.25\text{ }\mu\text{m}$ ($\Delta\lambda = 0.24\text{ }\mu\text{m}$), $1.65\text{ }\mu\text{m}$ ($\Delta\lambda = 0.23\text{ }\mu\text{m}$), $2.25\text{ }\mu\text{m}$ ($\Delta\lambda = 0.28\text{ }\mu\text{m}$) and $2.8\text{ }\mu\text{m}$ ($\Delta\lambda = 0.7\text{ }\mu\text{m}$). The field of view of each element was $0.3R_{\odot} \times 0.3R_{\odot}$, and the arrays were orientated perpendicular to the scan path.

A 15,000-m³ balloon was successfully launched by a joint team of ISAS (the Institute of Space and Astronautical Science, Japan) and LAPAN (Indonesian National Institute of Aeronautics and Space) at the Watukosek Balloon Base in East Java. Virtual tracking of the eclipsed Sun at a floating altitude of 30 km was performed by rotating the magnetometer stage at an appropriate rate after the desired instrument orientation had been obtained. The attitude stability was better than 10 arc s ($\sim 0.01R_{\odot}$). Absolute positional error was $<0.2R_{\odot}$ in the scan

direction and $<0.3R_{\odot}$ in the perpendicular.

Scanning was performed by small-amplitude oscillation around the cross-elevation axis. The width of the scan was 5° ($20R_{\odot}$) and its rate was 0.05 deg s^{-1} ; this allowed $1\frac{1}{2}$ complete scans between $6R_{\odot}$ on the west side of the Sun and $14R_{\odot}$ on the east side. Scan paths crossed the ecliptic plane on the west side at $\sim 4R_{\odot}$ with an inclination of $\sim 7^{\circ}$. The configuration of the scan path and beam positions of each detector are shown in Fig. 1. In-flight and post-flight calibrations have indicated possible errors of $\pm 30\%$ in absolute brightness determination. Polarization at $2.25\text{ }\mu\text{m}$ was also measured using a rotating half-wave plate and a fixed polarizer. The result of the polarization measurement will be described elsewhere.

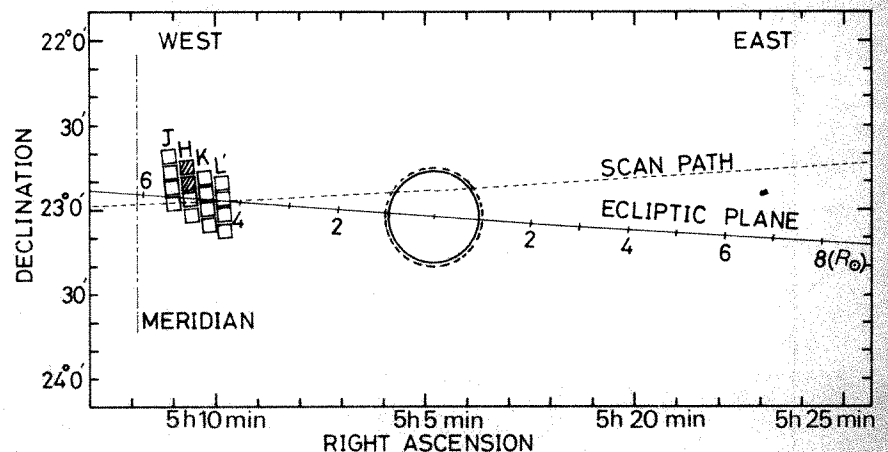
Figure 2 shows brightness distributions in each band on the west side of the Sun along the scan path. At $1.65\text{ }\mu\text{m}$, a definite excess over the smooth coronal component is seen between $2.8R_{\odot}$ and $4.5R_{\odot}$ from the Sun on the west side, showing a peak at $3.8R_{\odot}$. The brightness of the excess component at $3.8R_{\odot}$ on the west side is $6 \times 10^{-7}\text{ W cm}^{-2}\text{ }\mu\text{m}^{-1}\text{ sr}^{-1}$. A weaker excess is also seen between about $3R_{\odot}$ and $4.4R_{\odot}$ on the east side, where the peak again occurs at $3.8R_{\odot}$. The brightness of the excess component at $3.8R_{\odot}$ on the east side is $2.2 \times 10^{-7}\text{ W cm}^{-2}\text{ }\mu\text{m}^{-1}\text{ sr}^{-1}$. A relatively small but obvious excess emission is also seen in the $1.25\text{-}\mu\text{m}$ profiles from about $3.2R_{\odot}$ to $4.5R_{\odot}$ on the west side of the Sun, where scans have just crossed the ecliptic plane (see Fig. 1). The peak is again at $\sim 3.8R_{\odot}$ and the brightness of the excess component is $3.7 \times 10^{-7}\text{ W cm}^{-2}\text{ }\mu\text{m}^{-1}\text{ sr}^{-1}$. The contribution of stray light from the strong inner corona has been estimated from independent laboratory data for the off-axis rejection rate. The effect is not serious in the region further than $1.8R_{\odot}$ in $1.65\text{-}\mu\text{m}$ and $2.25\text{-}\mu\text{m}$ bands, or further than $3.0R_{\odot}$ in $1.25\text{-}\mu\text{m}$ and $2.8\text{-}\mu\text{m}$ bands. Any error due to such stray light is safely below 30% of the above excess emissions.

In the $2.25\text{-}\mu\text{m}$ band, we have failed to detect definite peaks larger than the 3σ detection limit of $1.2 \times 10^{-7}\text{ W cm}^{-2}\text{ }\mu\text{m}^{-1}\text{ sr}^{-1}$, owing to degraded sensitivity in this band. However, a small feature could be discerned at about $4R_{\odot}$ on the west side, where the brightness is $1 \pm 0.4(1\sigma) \times 10^{-7}\text{ W cm}^{-2}\text{ }\mu\text{m}^{-1}\text{ sr}^{-1}$, roughly consistent with the previous observations of Peterson¹ and MacQueen². We found no definite excess component in the $2.8\text{-}\mu\text{m}$ band at the 3σ level of $1.2 \times 10^{-7}\text{ W cm}^{-2}\text{ }\mu\text{m}^{-1}\text{ sr}^{-1}$.

Figure 3 shows a near-infrared energy distribution of the excess component obtained from the present observation, together with previous $2.2\text{-}\mu\text{m}$ data. It has a maximum at $1.65\text{ }\mu\text{m}$ and drops off at both shorter and longer wavelengths, especially between 1.65 and $2.25\text{ }\mu\text{m}$, differing significantly from the solar spectrum. Therefore, we argue that the excess emission component is not caused by scattered light but is due mainly to thermal radiation of circumsolar dust particles of appropriate composition to produce the spectrum.

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Fig. 1 The scan path of the telescope beam and the projected focal plane array of detectors. J, H, K and L' denote the 1.25-, 1.65-, 2.25- and 2.8- μm bands, respectively. Data from two detectors of the 1.65- μm band, indicated by hatched squares, were not used for data analysis, due to anomalous large noise.



Lamy has proposed⁶ that the near-infrared excess emission may be caused by silicate grains such as obsidian particles lying just outside a dust-free zone, while Mukai *et al.*⁷ have suggested a two-component model of obsidian and graphite. However, the spectral features observed by us are not consistent with these materials, even taking into account the large uncertainty in the data at longer wavelengths. One interpretation we have envisaged is that the emission in question is accounted for by thermal emission from olivine particles of the order of 100 μm in radius at a temperature of $\sim 1,300$ K. Olivine possesses a strong absorption band between 0.8 and 1.7 μm , and hence is more emissive within this band. Smaller olivine particles seem unlikely because the intensity of scattered light would obliterate the thermal radiation, making the spectrum appear solar. As an illustration, the thermal emission spectrum of olivine of radius 100 μm at a temperature of 1,300 K is shown in Fig. 3.

Our suggestion that the circumsolar dust may consist of relatively large particles composed mainly of olivine is based on recent studies of interplanetary dust and its mineralogy. Weiss-Wrana⁸ has shown that the principal properties of the zodiacal light ('the false dawn') are well represented by opaque irregularly-shaped chondritic particles in the size range 20–120 μm . Most stratospheric micro-meteorites, known as Brownlee particles⁹, have an opaque, black appearance and a composition similar to CI carbonaceous chondrites. Furthermore, olivine

is known to be a major constituent of extraterrestrial solids such as stony meteorites. Finally, Hashimoto *et al.*¹⁰ have demonstrated, in experiments on the thermal metamorphism of C2 type carbonaceous chondrites, that the matrix (mineralogically amorphous) becomes olivine when heated in a low pressure environment to $>1,000$ K.

The infrared spectrum we have observed can thus be interpreted as being due to the thermal metamorphism of dust particles as they spiral down to the hot region of the circumsolar dust cloud. However, the orbital radius at which the emission peaks were recorded must be explained in terms of a time-dependent vaporization process of relatively large particles (of the order of 100 μm) having optical properties that are susceptible to temperature changes—ranging from opaque in distant regions to a more transparent (olivine-like) nature in nearer and thus hotter regions around the Sun. A problem concerning the equilibrium temperature of such particles as envisaged here is that, according to Röser and Staude¹¹, a 100- μm olivine particle would have a temperature of 2,000 K at $\sim 4R_{\odot}$.

The present observations also provide information about the spatial distribution of dust, although the scan paths were such that a genuine central peak on the east side of the ecliptic plane was missed. As shown in Fig. 2, the excess component of the 1.65- μm band seems to extend from $2.8R_{\odot}$ to $4.5R_{\odot}$, with a small peak at around $3.8R_{\odot}$. (These features bear some

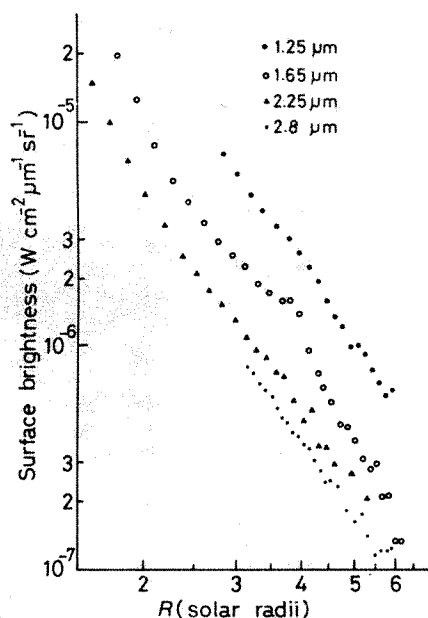


Fig. 2 Brightness distributions of each band observed on the west side of the Sun. The data are averaged over every $0.15R_{\odot}$ except for the 2.8- μm band inside $4.4R_{\odot}$, where the interval is $0.08R_{\odot}$.

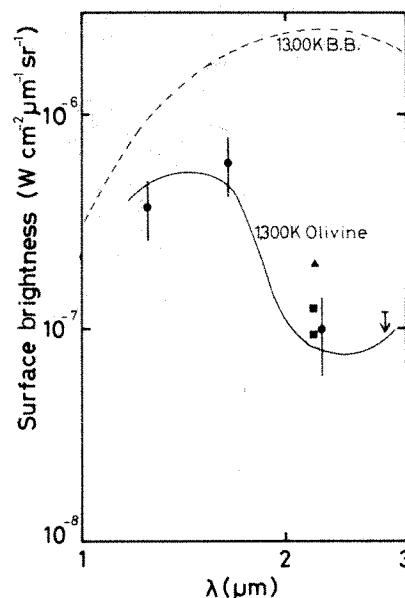


Fig. 3 Energy spectrum of the excess emission component. Previous observations at 2.2 μm are also shown: $\blacktriangle$, ref. 1; $\blacksquare$, ref. 2. The solid curve represents the thermal radiation of an olivine particle of radius 100 μm at a temperature of 1,300 K. The dashed line is a 1,300 K black body curve for comparison.

resemblance to the 2.2- μm profile found by Peterson¹², but differ in that our data show no steep trough just inside the narrow peak.) The 1.65- μm brightness also shows an excess emission between $3.0R_{\odot}$ and $4.4R_{\odot}$ in the east side scans, which are $\sim 1R_{\odot}$ from the ecliptic at $4R_{\odot}$, and its intensity is considerably smaller. This suggests that the circumsolar dust cloud is a ring and not a spherical shell and is consistent with the hypothesis that the dust particles responsible for the observed infrared excess emission are identical with the interplanetary dust causing the zodiacal light—which is generally assumed to be reflected by grains as large as a few hundred micrometres, probably in orbits of high ellipticity (see, for example, ref. 13).

Finally, we can make a rough estimate of the total mass of the dust cloud in the region of infrared emission. Let us assume that the cloud is formed into a doughnut shape of mean diameter $8R_{\odot}$, radial width $1R_{\odot}$ and thickness $1R_{\odot}$, and that the rep-

resentative radius of olivine particles is 100 μm . The observed infrared fluxes measured at 1.25 and 1.65 μm will then be related, as an order of magnitude consideration, to an average number density of 1×10^{-14} particles per cm^3 and a total dust mass of 7×10^{14} g. The spatial density obtained here is about 15 times the value of simple extrapolation based on the mass density 4×10^{-23} g cm^{-3} at 1 AU (ref. 14), applying an $r^{-1.3}$ dependence on heliocentric distance.

We thank Professor H. Hasegawa for his advice and encouragement, Drs H. Tanabe, S. Isobe and T. Mukai for useful discussion, Professor J. Nishimura, Messrs H. Akiyama, Y. Koma and Y. Okabe of ISAS for their cooperation in this balloon programme, and all the staff of the balloon team of LAPAN, headed by Drs J. Soegijo and T. E. Hariadi. This research was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan.

Received 18 June; accepted 24 August 1984.

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New model of Saturn's ionosphere with an influx of water from the rings

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Current theoretical models of Saturn's ionosphere are similar to those of Jupiter's because of the gross similarity of their upper atmospheres. In the case of Jupiter, the theoretical models can be fitted reasonably well to ionospheric electron density profiles^{1,2} obtained from the Pioneer and Voyager radio occultation experiments³⁻⁵. In contrast, the theoretical models of Saturn's ionosphere are inconsistent with both the ionospheric electron density profiles^{6,7} obtained from the Pioneer and Voyager occultation observations⁸⁻¹⁰ and the large diurnal variation of maximum ionospheric electron density deduced from studies of Saturn lightning discharges^{11,12}. We propose a radically different model of Saturn's ionosphere in which water plays a major role as a minor constituent present by downward diffusion from an external source. Our model Saturn ionosphere is a classical 'F2' type layer resulting from the photodissociative production of H^+ from H_2 and rapid chemical loss by a series of charge exchange reactions with water. A planet-wide influx of $\sim 4 \times 10^7$ molecules $\text{cm}^{-2} \text{s}^{-1}$ of water from the rings is consistent with the observed ionospheric electron densities. An enhanced influx of water occurs at latitudes (-38° , $+44^\circ$) connected magnetically to the inner edge of Saturn's B ring¹² where an electromagnetic erosion process¹³ takes place. Present-day influx at these latitudes may be as large as $\sim 2 \times 10^9$ molecules $\text{cm}^{-2} \text{s}^{-1}$.

The essential features of previous theoretical models of Saturn's ionosphere are summarized below. The major topside ion, H^+ , is produced via photoionization of atomic H or photodissociative ionization of H_2 . Both H and H^+ are extremely long-lived above the homopause ($\sim 1,100$ km altitude; all altitudes referenced to the 1 bar pressure level) and are removed

Table 1 Chemical reactions in Saturn's ionosphere: H_2 , H_2O , H

	Ion production	Rate*	Ref.
A1	$\text{H}_2 + h\nu \rightarrow \text{H} + \text{H}$	$4.5 \times 10^{-10} \text{ s}^{-1}$	[7]
A2	$\text{H}_2^+ + \text{e}$	5.4×10^{-10}	[7]
A3	$\text{H} + \text{H}^+ + \text{e}$	9.5×10^{-11}	[7]
A4	$\text{H} + h\nu + \text{H}^+ + \text{e}$	7.3×10^{-10}	[7]
A5	$\text{H}_2\text{O} + h\nu \rightarrow \text{OH} + \text{H}$	1.0×10^{-7}	[19]
	$\text{OH}^+ + \text{H} + \text{e}$	5.5×10^{-10}	[19]
	$\text{H}_2 + \text{O}(^1\text{D})$	1.4×10^{-8}	[19]
	$\text{H}_2\text{O}^+ + \text{e}$	3.3×10^{-9}	[19]
	$\text{H}_2 + \text{O}^+ + \text{e}$	5.8×10^{-11}	[19]
	$\text{H}^+ + \text{OH} + \text{e}$	1.3×10^{-10}	[19]
Charge exchange/recombination			
B1	$\text{H}_2\text{O} + \text{H}^+ \rightarrow \text{H}_2\text{O}^+ + \text{H}$	$8.2 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$	[18]
B2	$\text{H}_2\text{O}^+ + \text{H}_2 \rightarrow \text{H}_3\text{O}^+ + \text{H}$	6.1×10^{-10}	[18]
B3	$\text{H}_3\text{O}^+ + \text{e} \rightarrow \text{H}_2\text{O} + \text{H}$	4.3×10^{-7}	[18]
B4	$\text{H}_2 + \text{OH}$	4.3×10^{-7}	[18]
B5	$\text{H} + \text{H} + \text{OH}$	4.3×10^{-7}	[18]
B6	$\text{H}_2^+ + \text{H}_2 \rightarrow \text{H}_3^+ + \text{H}$	2.0×10^{-9}	[7]
B7	$\text{H}_2^+ + \text{e} \rightarrow \text{H}_2 + \text{H}$	1.0×10^{-7}	[7]
B8	$\text{H}^+ + \text{e} \rightarrow \text{H} + h\nu$	2.0×10^{-12}	[7]
B9	$\text{OH} + \text{H}_2 \rightarrow \text{H}_2\text{O} + \text{H}$	3.6×10^{-11}	[18]

* Photoionization rates normalized to 10 AU by $1/R^2$ scaling of reference values.

by downward diffusion to the vicinity of the homopause where appreciable concentrations of heavier gases (most importantly methane) are encountered. H^+ ion loss occurs essentially at the homopause via charge exchange and electron-ion recombinations, resulting in a peak ion concentration of $\sim 2 \times 10^5 \text{ cm}^{-3}$ at $\sim 1,200$ km altitude. This very simplified picture emerges from a consideration of many possible chemical reactions and detailed calculations taking into account the production, diffusion and chemical losses of many atmospheric constituents⁷.

Figure 1 is a representative summary of the dawn and dusk electron density profiles (as a function of altitude) obtained from analyses of the Pioneer and Voyager radio occultation data. In Fig. 1, mid-latitude Voyager observations are compared with a typical model ionospheric electron density profile. (Figure 2 shows all of the radio science observations.) The measured electron densities are all approximately an order of magnitude less than those calculated on the basis of current models.

Maximum observed ionospheric densities are $\sim 1 \times 10^4 \text{ cm}^{-3}$, compared with a calculated maximum density of $2 \times 10^5 \text{ cm}^{-3}$ in all previous models. The peak electron density is observed at between 2,000 and 3,000 km altitude instead of the calculated 1,200 km altitude. The discrepancy in the altitude of the peak is particularly significant in light of the thermal structure of the atmosphere. Above $\sim 1,600$ km, the atmosphere is isothermal, with a temperature of ~ 800 K and an atmospheric scale height of ~ 400 km. Below that the temperature decreases linearly to ~ 125 K at 1,000 km altitude where the atmospheric scale height is ~ 60 km (ref. 7). The difference in the observed and modelled altitudes of peak ion density is thus many atmospheric scale heights. The large (~ 2 orders of magnitude) diurnal variation of maximum ionospheric electron density (Fig. 3) deduced from analyses of Saturn lightning discharges^{11,12} is even more difficult to understand on the basis of current models of Saturn's ionosphere. Without an additional loss mechanism for rapid removal of the long-lived H^+ ions, no diurnal variation is expected since the time constant for loss of H^+ above the homopause is ~ 400 h that is, many planetary rotations. This is owing to the slow electron recombination reaction $\text{H}^+ + e \rightarrow \text{H} + h\nu$ at $2 \times 10^{-12} \text{ cm}^{-3} \text{ s}^{-1}$ and the large diffusion time constant below the 2,500 km altitude of the ionospheric peak.

The observational results thus require an additional and effective loss mechanism for H^+ ions, since ion production via photodissociative ionization of H_2 and photoionization of atomic H is well understood. Several possible loss processes have been quantitatively evaluated but none has proven adequate. These include losses due to an increased methane abundance which requires an unreasonably high eddy diffusion coefficient¹⁴, removal of H^+ by vibrationally excited H_2 (ref. 6), and/or the presence of large vertical drifts^{7,8}. The possibility of an interaction between the ionosphere and ring system was recognized as early as 1975¹⁵ and a suggestion involving H^+ ion loss due to the presence of OH radicals from the rings^{16,17} was made to explain the low ionospheric densities inferred from the Pioneer observations.

We propose a simple model ionosphere dominated by the removal of H^+ ions via charge exchange with water and subsequent electron recombination. Table 1 summarizes the important chemical reactions and reaction rates; the reactions involving water have been largely drawn from work relating to comet models^{18,19}. The important reactions are H^+ ion production via dissociative photoionization of H_2 (A3) and loss via the charge exchange with H_2O (B1). The H_2O^+ ion is very short lived, reacting with H_2 (B2) to produce H_3O^+ , which is then readily removed by electron recombination (B3-B5). As in previous models, the H_2^+ ions produced by photoionization of H_2 (A2) are rapidly removed via reaction with H_2 (B6) and charge exchange (B7) and are not a major topside ion. The direct photoionization of atomic hydrogen (A4) does not significantly contribute to the H^+ ion density owing to the limited availability of atomic H.

The OH produced in B4 and B5 has a short lifetime ($\tau < 1$ s) at the altitude of the peak ion density owing to a reaction (B9) with H_2 , producing H_2O and H. Thus water acts as a catalyst in the rapid removal of H^+ ; each H^+ ion removed returns, on average, 3 or 4 H atoms with no associated chemical loss of H_2O . Photodissociation of H_2O (A5) yields OH which in the presence of appreciable H_2 (B9) rapidly reforms H_2O (as would OH from any other source). There is no net loss of water due to charge exchange or photoionization. Thus the density of water above the homopause is given by²⁰

$$N_w = N_0 \exp(-9Z/H) + FH_0/8D_0 [\exp(-Z/H) - \exp(-9Z/H)]$$

where N_0 , H_0 , and D_0 are the H_2O density, the atmospheric scale height, and molecular diffusion coefficient at a reference ($Z=0$) altitude, F is the downward flux of H_2O and H is the atmospheric scale height. Assuming that the water is readily removed by freezing out just above the homopause or by mixing

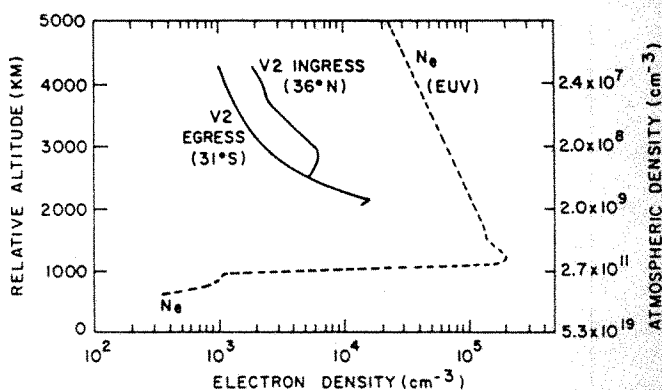


Fig. 1 Model calculations of mid-latitude ionospheric electron density as a function of altitude (extreme ultraviolet, EUV) and Voyager observations of Saturn's ionosphere at mid-latitudes, adapted from Atreya *et al.*⁷.

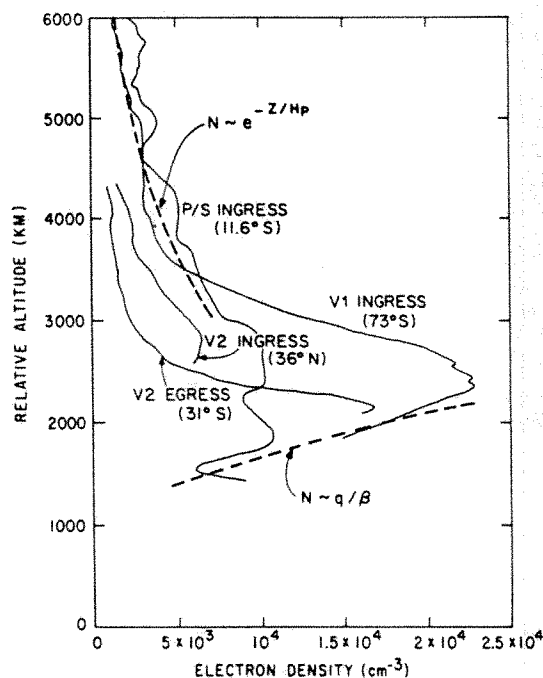


Fig. 2 Ionospheric electron density profiles deduced from Voyager 1 and 2 and Pioneer radio science data, adapted from Atreya *et al.*⁷. Altitude is referenced to the 1 bar pressure level.

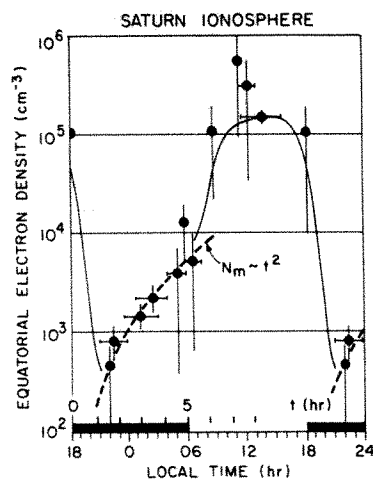


Fig. 3 Diurnal variation of maximum ionospheric electron density inferred from Voyager observations of lightning-generated radio emissions in Saturn's atmosphere, adapted from Kaiser *et al.*¹². The dashed line indicates a model fit to the nighttime increase of maximum ionospheric electron density.

and chemical loss at the homopause, the density of water throughout the (isothermal) region of interest above 1,600 km altitude is just $N_w = (FH/8D_0) \exp(-Z/H)$. Since the diffusion coefficient $D = D_0/N$, where N is the atmospheric density, the downward flux of water results in a water density proportional to the density of the H_2 atmosphere through which it flows under the condition of constant flux, that is, $N_w/N = \text{constant} = FH/8N_0D_0$.

Under these conditions a classical 'Bradbury layer', analogous to the Earth's F2 layer can form at an altitude where the chemical τ_c and diffusive τ_d time constants are comparable^{20,21}. For such a layer, the ionospheric peak forms above the level of maximum ion production owing to the upward diffusion of ions. Below that altitude the increasing chemical loss (charge transfer with water) effectively removes the major ion, so that most of the ionization anticipated by previous models is lost. The loss coefficient is $\beta = k[H_2O]$ where k is the rate coefficient of reaction B1 in Table 1. The required water concentration at the altitude of the ionospheric peak can be estimated by $N_m \equiv q(h_m)/\beta(h_m)$ where N_m is the maximum electron density at the altitudes (h_m) of the ionospheric peak, and the ion production function q and loss β are evaluated at the altitude of the peak. Taking $h_m \sim 2,500$ km, $q \sim 1 \text{ cm}^{-3} \text{ s}^{-1}$ and $N_m \sim 10^5 \text{ cm}^{-3}$, appropriate to the equatorial ionosphere at noon, yields a water concentration of $\sim 1,250 \text{ cm}^{-3}$ at 2,500 km altitude. This requires a $4 \times 10^7 \text{ cm}^{-2} \text{ s}^{-1}$ downward flux of water molecules. The maximum densities observed at the dawn and dusk terminators where the extreme ultraviolet production rate is ~ 0.1 of the maximum are ~ 1 order of magnitude lower than the noon density, as expected. Equating the chemical and diffusive time constants ($1/\beta \sim H^2/D$) yields an expected peak altitude between 2,000 and 2,500 km.

The variation of electron density with altitude can also be understood using simple approximations to 'Bradbury layer' behaviour that have been widely used in the study of the Earth's F2 layer^{20,21}. The ion distribution well above the peak is controlled by ambipolar diffusion, so $N \sim \exp(-Z/H_p)$ where H_p is the plasma scale height. This distribution is indicated in Fig. 2 assuming 800 K electron and ion temperatures, for which $H_p = 4H$. The H^+ ion density distribution below the peak is controlled by chemical loss, with $N(h) \sim q/\beta(h)$. The ion production, q , varies slowly, so, to first approximation, we expect $N(h)$ below the peak to increase with height as $\exp(h/H)$, as is illustrated in Fig. 2. This reflects the increased loss at greater depths due to greater water concentration. The peak has a width of a few scale heights and occurs at an altitude (2,500 km) where the diffusion time constant is a few hours, as does the Earth's F2 layer. However, the rapid nighttime decay of the equatorial ionospheric density, evidenced in Fig. 3, requires a more rapid loss, perhaps a downward drift related to the ionosphere-protonosphere exchange described below.

Figure 3 shows an ionospheric density minimum just after dusk and a steady increase in near-equatorial ionospheric density throughout the night. We attribute this phenomenon to a diurnal ionosphere-protonosphere exchange similar to that proposed for the Earth's nighttime F2 layer²⁰. During daylight hours, photoproduction in Saturn's ionosphere pumps protons outward along field lines to great heights. At night, a relaxation back towards the ionosphere occurs to replenish the nighttime ionospheric density. If we assume a uniform density (n) column of protons at rest at dusk, under constant downward acceleration (a) the proton influx $\Phi_p = nat$ increases linearly with time. Supposing the nighttime maximum ionospheric density occurs at high altitudes where loss is negligible, the increase in the maximum density with time is related to the influx by $N_m(t) = \int \Phi_p/H dt = (na/2H)t^2$. This increase is indicated in Fig. 3 by the dashed line, which fits the nighttime observations well with $na/2H \sim 2 \times 10^{-5} \text{ cm}^{-3} \text{ s}^{-2}$, corresponding to a proton influx increasing to a maximum of $\sim 2 \times 10^7 \text{ cm}^{-2} \text{ s}^{-1}$ just before dawn. The equivalent ion production rate at dawn due to this exchange ($\sim 0.2 \text{ cm}^{-3} \text{ s}^{-1}$) is comparable to or greater than the extreme ultraviolet production. Thus, we envision a very dynamic ionos-

phere, characterized by ion production and transport into a vast protonosphere reservoir by day, coupled with relaxation and return by night.

The increased ion losses at latitudes connected magnetically to the inner edge of Saturn's B-ring¹² can be explained by an increased local water influx of as much as 2×10^9 molecules $\text{cm}^{-2} \text{ s}^{-1}$. The erosional process described by Northrop and Hill¹³ provides a mechanism whereby sub-micrometre charged particles (agglomeration of water molecules), unstable in Saturn's ring plane, precipitate along magnetic field lines into the ionosphere. If these particles are deposited directly at the level of the ionospheric peak ($\sim 2,500$ km) the required flux is less than $2 \times 10^9 \text{ cm}^{-2} \text{ s}^{-1}$. At this maximum flux, the ring mass loss is $\sim 6 \times 10^{-14} \text{ g cm}^{-2} \text{ s}^{-1}$. The large dayside extinction was observed at latitudes of -37 to -39° (ref. 12), the maximum southerly extent of the Voyager 1 spacecraft. Averaged over $\sim 4^\circ$ latitude extent of the presumed source region in the rings ($1.52 - 1.62 R_s$), the total ring mass loss is $\sim 1.2 \times 10^6 \text{ g s}^{-1}$ or $1.6 \times 10^{12} \text{ g yr}^{-1}$, comparable to the estimated total mass influx from interplanetary meteoroids²². At this rate, 60 g cm^{-2} of material can be removed from the source region in $\sim 30 \times 10^6$ years, suggesting that this process may be responsible for the large decrease in optical depth of the rings inward of $1.62 R_s$.

The Pioneer and Voyager observations of Saturn's ionosphere and the inferences drawn from studies of Saturn lightning can be largely understood on the basis of a new model of Saturn's ionosphere in which water plays a major role. The planetwide influx of water (4×10^7 molecules $\text{cm}^{-2} \text{ s}^{-1}$) required is approximately an order of magnitude greater than that expected from photosputtering of the rings²³. Morfill *et al.*²² estimate that micrometeorite impacts on Saturn's icy rings produce between 0.75 and $6 \times 10^6 \text{ g s}^{-1}$ of vapour, $\sim 10\%$ of which is deposited in Saturn's atmosphere. This results in an estimated influx of between 0.6 and 4.5×10^7 molecules $\text{cm}^{-2} \text{ s}^{-1}$. In spite of the uncertainties in this estimate²², micrometeorite bombardment of Saturn's rings is an attractive source mechanism because it provides an explanation of the vast differences between Jupiter's and Saturn's ionospheres. An atmospheric influx of water has been previously suggested to explain features of ultraviolet spectra of Saturn²⁴ and Voyager infrared spectra of Titan²⁵. The increased influx of water at latitudes magnetically connected to the inner edge of the B-ring is not inconsistent with expected erosion rates. Finally, we infer a diurnal ionosphere-protonosphere exchange in which protons pumped into the protonosphere by day return by night.

Received 4 June; accepted 10 September 1984.

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Production of ammonia-depleted surface layers on the saturnian satellites by ion sputtering

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It is generally believed that some type of plains-forming resurfacing has continued into the recent geological past on the surfaces of the saturnian satellites, particularly Enceladus¹⁻³. In conditions of tidally-driven heating, 'magma' could emerge and produce the plains especially evident on this satellite, as well as on Tethys, Dione, and Rhea. The magma favoured at present, considering the temperatures of the satellites and their likely formation, is the peritectic melt $2\text{H}_2\text{O}\cdot\text{NH}_3$ (refs 1-4). Surprisingly, however, visible and near-IR spectra have not shown any evidence of ammonia on the optical surface of any of the satellites, although absorption bands indicative of water ice are abundantly in evidence. On the basis of laboratory-derived data on the ion erosion of water and ammonia ices, we suggest that the satellite surfaces could have become enriched with water ice over a reasonable geological time period (10^6 – 10^9 yr) owing to preferential erosion of the ammonia by magnetosphere ion bombardment. This mechanism would be an alternative explanation to other processes invoked for the absence of ammonia spectral features. The concept is similar to that of Haff⁵ who has proposed that a crust of non-volatiles could eventually coat the Galilean satellites J3 and J4 (Ganymede and Callisto) through preferential water ice erosion (an 'armouring' process).

Sputtering rates for water ice expected on the four saturnian satellites Enceladus, Tethys, Dione and Rhea, using laboratory-measured H_2O ice erosion rates⁶ and Voyager-measured ion fluxes⁷ ≥ 30 keV in the vicinity of the satellites have recently been reported⁸. These rates are shown in Table 1, together with the expected surface loss of water ice in $\text{cm } 10^{-9} \text{ yr}^{-1}$. The results are given for Voyager 2 measured particle fluxes at Enceladus (the Voyager 1 trajectory did not go in to this distance) and Voyager 1 measured fluxes at the other three moons. The rates derived from the Voyager 2 fluxes are approximately a factor of 20 less than the Voyager 1 derived values, reflecting the temporal changes in the magnetosphere plasma population. The uncertainties in the laboratory numbers are $\sim 20\%$ (ref. 6). The surface loss determination takes into account the escape fractions expected from the satellites under gravitational forces, using laboratory-determined velocity spectra for the eroded molecules⁹. (For example, 80–90% of the eroded molecules are expected to escape Enceladus.) These molecules, sputtered from the satellites as well as the E-ring, could produce the oxygen torus around the planet.^{10,11} Sputtering of the satellite surfaces by lower-energy, corotating ions may increase the rates and the erosion depths given in Table 1. In contrast, a small atmosphere or magnetic field on any of the moons would exclude lower-

energy plasma ions from striking a satellite surface. Thus, the values in Table 1 are probably a conservative estimate of the surface effect.

The values of Table 1 indicate that a surface loss of water ice ranging from a few centimetres to several metres could be expected on Enceladus, depending on the composition of the ion-flux in the vicinity of the satellite. Determinations of this composition from the Voyager 2 data suggest that there is an appreciable flux of energetic oxygen in the vicinity of Dione and Enceladus⁷. Thus, the higher values of the erosion rate and the net erosion are probably more representative of the actual situation. The erosion values for Table 1 were calculated assuming isotropic ion bombardment of the satellites, because the pitch angle anisotropies are sufficiently small in this region of the magnetosphere⁷.

We have determined in the laboratory the erosion yield Y of frozen ammonia molecules (at $\sim 10\text{K}$) under bombardment by 1.5-MeV He^+ and Ne^+ ions. These values are $Y(\text{He}^+) \sim 140$ molecules per incident ion and $Y(\text{Ne}^+) \sim 2 \times 10^3$ molecules per incident ion. At this temperature in these experiments we found no evidence for the preferential removal from the surface of the hydrogen compared with the nitrogen. However, the NH_3 could be released as atoms and/or molecules other than ammonia, as has been reported in the case of frozen NH_3 bombarded by 3-keV argon ions¹² (absolute yields are not determined in this experiment). The $Y(\text{He}^+)$ on NH_3 is comparable with those we have measured from solid oxygen and solid carbon dioxide at these temperatures, and is ~ 18 times the erosion yield on water ice (~ 8).

The detailed erosion yield studies of water ice in the laboratory show that at temperatures $\leq 100\text{K}$, for fast light ions ($\geq 1\text{keV AMU}^{-1}$), the yield varies roughly as the square of the electronic stopping power of the incident ion⁶. The ratio of the 1.5-MeV He^+ and Ne^+ erosion yields on NH_3 also varies as roughly the square of the ratio of the stopping powers. We have previously found that the erosion of water ice by heavy ions depends more steeply than quadratically on the stopping power^{6,13}. From data on heavy ions incident on H_2O ice, we have $Y(1.5\text{-MeV O}^+) \sim 650$. The stopping power of neon and oxygen are quite similar at these energies, so that we can confidently use the laboratory values for $Y(\text{Ne}^+)$ in the following.

If we assume that the molecular species in a water ice-ammonia mixture sputter at a rate proportional to the individual sputtering yields, the equilibrium concentration of ammonia is $\sim 5\%$ for light-ion (proton) irradiation to $\sim 30\%$ for only heavy-ion (oxygen) bombardment. At the higher temperatures (but $< 100\text{K}$) expected on Enceladus, the ammonia fraction will be lower as the yield for the more volatile NH_3 is expected to increase with increasing temperature. Such temperature dependencies of the yield have been observed in the range 50–90 K for CO_2 and SO_2 (ref. 6), which have material binding energies bracketing that of NH_3 . At temperatures approaching the NH_3 sublimation temperature, more volatile species, such as N_2 and H_2 , are likely to be produced¹², as in the case of sputtering of H_2O ice⁶.

As the ion bombardment enhances diffusion to the surface of the more volatile species, the layer bombarded will be depleted in nitrogen down to the penetration depth of the incident ions (~ 0.3 – $3\text{ }\mu\text{m}$ for 10 – 10^3 keV protons and a tenth that for incident O^+). In fact, much deeper layers of ammonia depletion might be expected for several reasons. Ion erosion produces a very non-uniform surface and other geological processes (such as internal heating) and exogenic processes (such as micrometeoroid gardening) can bring fresh material to the surface. Also, any ice grains from the E-ring which might coat the surface will be depleted in ammonia by ion bombardment.

Taking the values of the erosion for ammonia ice estimated above, we find that for a pure ammonia ice surface the ammonia removal on Enceladus would be some tens of centimetres (erosion by protons) to tens of metres (erosion by oxygen ions) in 10^9 yr (Table 1). Hence, in 10^9 yr , it would not be unreasonable to expect the ammonia in a frozen magma to be eroded away.

Table 1 H_2O ice erosion

Satellite	Escape flux ($10^8 \text{ H}_2\text{O cm}^{-2} \text{ s}^{-1}$)		Surface loss ($\text{m } 10^{-9} \text{ yr}^{-1}$)	
	Protons incident	Oxygen incident	Protons incident	Oxygen incident
Enceladus*	0.01	1.0	0.01	1.0
Tethys†	0.05	4.0	0.06	4.0
Dione†	0.06	5.0	0.08	6.0
Rhea†	0.02	2.0	0.02	2.0

* Voyager 2 derived numbers.

† Voyager 1 derived numbers.

leaving the surface rich in water ice. If the resurfacing of the plains of Enceladus occurred $\sim 10^6$ yr ago, although the amount of ammonia eroded would be much less, enough modification would occur to prevent the detection of ammonia in the infrared. We also note that estimates of erosion over long time periods must take into account possibilities of variations in magnetosphere fluxes. The absence of reports of detection of ammonia in the magnetosphere does not indicate the absence of ammonia and/or a nitrogen by-product. In these regions, the cold- and hot-plasma instruments could not distinguish clearly between nitrogen and oxygen. Further, nitrogen from dissociated ammonia would be indistinguishable from a nitrogen contribution from Titan's atmosphere.

A process complementary to that discussed here can result from micrometeorite impacts on the satellite surfaces. Although not discussed in detail here (micrometeoroid bombardment is reviewed in ref. 14), note that such an impact will probably be enhanced by 'gravitational focusing' around Saturn, compared with the rates expected in the heliospheric ecliptic plane (for example, on the moons of Earth and Mars). NH_3 and H_2O have vastly different vapour pressures; thus, the vaporization and

fractionization produced by micrometeoroid impact would tend to deplete the surfaces of NH_3 preferentially.

These considerations present the intriguing possibility that, under an assumption of known magnetosphere charged-particle fluxes, an estimate can be made of the lower limit of the age of the last major satellite resurfacing. Furthermore, knowledge of the ammonia erosion rate can place an upper limit on the rate of continual resurfacing of a satellite surface, if that is a viable 'tectonic' process, in contrast to sudden major occurrences of resurfacing. This coupling of geological processes with space plasma physics processes is an exciting prospect, as for the case of Io's contribution to Jupiter's heavy ion magnetosphere torus.

We thank Dr W. B. McKinnon for discussion, and other participants in the NATO Research Workshop on Ices in the Solar System, where some of these ideas were generated. We thank Dr S. Squyres for helpful comments on the first version of this paper; L.J.L. also thanks Dr D. J. Stevenson for a helpful discussion. The work at the University of Virginia was supported in part by the NSF Astronomy Division under grant No. AST-82-00477 and the NASA Geophysics/Geochemistry Division under grant NAGW-186.

Received 13 July; accepted 24 August 1984.

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Midpoint scission of macromolecules in dilute solution in turbulent flow

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Drag reduction by polymer additives is commonly explained in terms of nearly complete extension of the macromolecules by high extensional strain rate regions of irrotational flow associated with turbulent bursting¹⁻⁵. The intermittent nature of the bursting process means that the polymer conformation cannot be measured directly by optical methods such as light scattering or birefringence. We have studied the degradation of narrow molecular weight distribution (MWD) polystyrene in a turbulent flow device which has been carefully constructed to minimize all sources of extensional flow except for that encountered in the turbulent flow itself. We report here the results of size exclusion chromatography of the degraded samples which show that chain scission occurs in a narrow distribution around the chain midpoint, indicating that the macromolecules are highly extended when they break. This represents the most convincing evidence yet for high chain elongation of polymers in a turbulent flow.

High chain elongation has been shown to occur in model laminar extensional flows by birefringence^{2,6}. It has also been shown that laminar extensional flow induces scission of polymers in a fairly narrow distribution centred on the chain midpoint^{6,7}. Midpoint scission itself is evidence of high chain extension since the highly elongated state is the only conformation of a randomly coiling macromolecule that would cause the maximum hydrodynamic force to be at the centre of the contour length of the chain.

The degradation studies were done in a single-pass gas pressure-driven flow device (see Fig. 1 legend for a description of the device). To perform a degradation run, the degraded sample collection reservoir and flow tube were filled with pure solvent and the feed solution reservoir was filled with the polymer solution. The system was pressurized to 20-87 atm and the

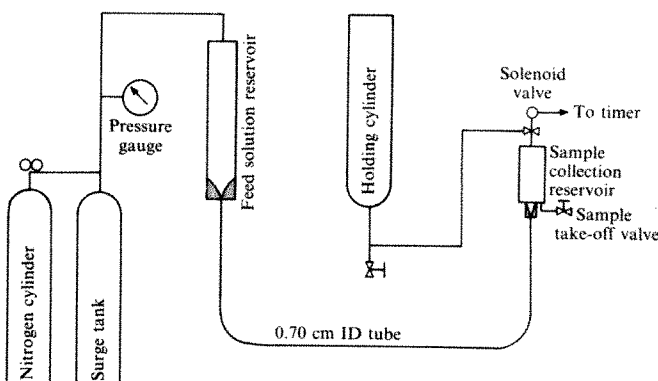


Fig. 1 Schematic diagram of the turbulent flow degradation apparatus. The apparatus comprises two reservoirs of 8.9 cm ID (internal diameter) connected by a 0.70-cm ID stainless steel tube 244 cm long. The entrance to the tube from the feed solution reservoir comprises a conical funnel machined on a 4.1 cm radius that smoothly reduces the diameter from that of the reservoir to that of the tube. (This lowered by an order of magnitude the extensional strain rates caused by the entrance, and was necessary as Culter *et al.*¹⁰ have shown that entrance effects can dominate polymer degradation even in turbulent flow.) The exit from the tube to the degraded sample collection reservoir consists of a cone of 7° angle which reduces the fluid velocity by an order of magnitude. A timer-controlled solenoid valve is mounted downstream of the degraded sample collection reservoir, and solution leaving the solenoid valve is sent to a holding cylinder. A sampling valve is mounted on the degraded sample collection reservoir upstream of the solenoid valve.

solenoid valve was opened for 1.5-4 s. Polymer solution passed through the degraded sample collection reservoir and solenoid valve to the holding cylinder. While the concentration of the polymer solution changed in the degraded sample collection reservoir during the experiment, the MWD was always that of the polymer that had experienced the turbulent flow.

The molecular weight distributions of the samples were determined using size exclusion chromatography (SEC). The system consisted of a pair of Waters Ultrastaygel (10^5 Å and 10^6 Å

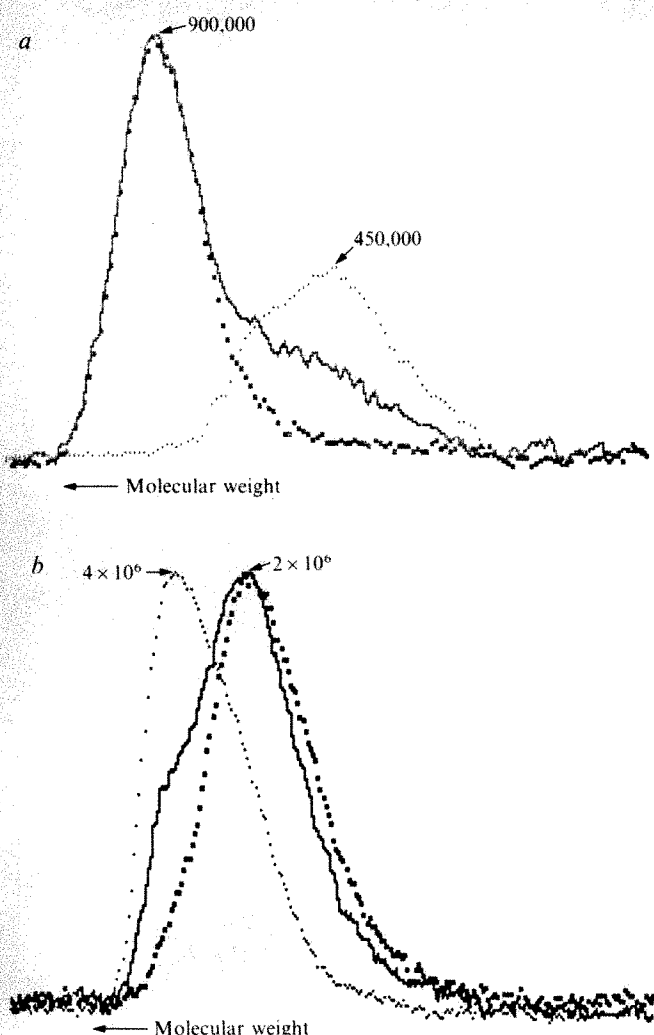


Fig. 2 *a*, Chromatograms of original (---) and degraded (—) 900,000-MW polystyrene. ····, 2.5 times the difference (degraded - original). *b*, Chromatograms of original (····) and degraded 3.84 × 10⁶-MW polystyrene. —, Degraded sample (single pass at 21 atm total pressure drop); ---, degraded sample (single pass at 40 atm total pressure).

pore size) columns; the plate count for the system was greater than 30,000 plates. Anionically polymerized polystyrene of molecular weight (MW) 900,000 (ratio for weight average MW to number average = 1.04 by SEC) was obtained from Pressure Chemical Co. and a sample of MW 3.84 × 10⁶ was purchased from Toyo Soda Ltd. The 3.84 × 10⁶-MW sample had a dispersity of 1.15 by SEC. The degradations were done at a polymer concentration of 50 p.p.m. in chloroform. The shear viscosity of the solution was identical to that of the pure solvent.

Figure 2*a* shows chromatograms of undegraded 900,000-MW polystyrene and the same sample after a single pass through the apparatus at 87 atm total pressure drop (Reynolds number = 730,000 and the wall shear stress was estimated as 3,000 N m⁻²). The height of the chromatogram has been normalized to simplify comparison. The degraded sample exhibits a shoulder centred at MW 450,000. When the chromatogram of the original sample is subtracted from that of the degraded sample (normalized according to peak area), the difference curve peaks at MW 450,000 (Fig. 2*a*). This proves that scission of the macromolecules occurs in a fairly narrow distribution around the chain midpoint. Midpoint scission is also confirmed by the data for the 3.84 × 10⁶-MW polymer in Fig. 2*b*. The chromatogram of the undegraded sample consists of a single peak at MW 4 × 10⁶; for the sample degraded at a system pressure drop of 21 atm, there is a shoulder at MW 4 × 10⁶ due to the unbroken fraction and a peak centred at MW 2 × 10⁶ due to the

degraded material. At 40 atm pressure drop, essentially all of the original polymer is degraded and there is a single peak centred at MW 2 × 10⁶ (Fig. 2*b*). These data are consistent with midpoint scission governed by a rate constant which decreases with decreasing molecular weight.

By using simultaneous birefringence and degradation measurements in a cross-slot flow device, Odell *et al.*⁶ have shown that midpoint scission occurs when the macromolecule is fully extended in an extensional flow. Recent SEC degradation studies done in our laboratory with a sudden contraction device confirm midpoint scission of polymer chains in extensional flow. As discussed in ref. 7, we model the extended polymer chain as a string of beads aligned with the flow to calculate the drag force on the chain. Following Zhurkov⁸, main-chain bond scission of a bond in tension is modelled as a first-order kinetic process with the rate constant given by

$$k_i = A \exp [-(E_a - F_i \Delta r) / kT] \quad (1)$$

where k_i is the first-order rate constant for scission of the i th bond, E_a is the bond energy, F_i is the force on that bond, Δr is the distance a bond must be separated to break, and k is Boltzmann's constant. Using this model, drag forces obtained from curve fitting of experimental data are of the correct order of magnitude. The width of the second peak is correctly predicted by equation (1). These results, taken in conjunction with the data of Odell *et al.*⁶, provide convincing evidence that full chain extension does indeed occur in extensional flow before chain scission and that this leads to scission centred on the chain midpoint.

Thus, the data presented here, showing midpoint scission of macromolecules in turbulent flow, strongly imply that the polymer chains can indeed be fully extended by turbulent flow, as well as by laminar extensional flow. Previous studies have suggested that scission is centred on the midpoint⁹; this notion was based primarily on the observation that the molecular weight distributions of broad MWD polymers are narrowed by degradation. The chromatograms of the degraded narrow MWD samples presented here provide the most direct evidence that we are aware of for high extension of randomly coiling macromolecules in turbulent flow.

We thank the Arco Chemical Co. for their support of this project.

Received 18 April; accepted 21 August 1984.

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Ocean-atmosphere coupling over monsoon regions

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In monsoon regions, the seasonal migration of the intertropical convergence zone (ITCZ) is manifested as a seasonal reversal of winds. Most of the summer monsoon rainfall over India occurs owing to synoptic and large-scale convection associated with the continental ITCZ (Fig. 1). We have investigated the interaction between these large-scale convective systems and the ocean over which they are generated¹⁻³, concentrating on the relationship between organized convection over the Indian Ocean and sea surface temperature (SST). We report here that on a monthly basis

the degree of cloudiness correlates well with SST for the relatively colder oceans, but when SST is maintained above 28 °C it ceases to be an important factor in determining the variability of cloudiness. Over the major regions of convection east of 70° E, which are warm year after year, the observed cloudiness cannot be correlated with variations in SST.

Organized convection over the equatorial Indian Ocean has an important role in maintaining the continental ITCZ¹⁻³ (Fig. 2), and the degree of continental cloud cover is linked to that over the ocean. The most important factor governing generation and maintenance of synoptic and large-scale cloudiness in the tropics is SST^{4,5}. Variability of large-scale convection over the Pacific has been shown to be intimately related to variations in SST^{6,7}. However, the coupling between the Indian Ocean and the continental ITCZ is more complex, involving correlations between the SST and oceanic cloudiness on the one hand and the oceanic and continental cloudiness on the other.

Not surprisingly, studies of the relationship between monsoon rainfall over India and the SST anomalies over different parts of the Indian Ocean, Arabian Sea and Bay of Bengal have yielded rather low correlation values⁸⁻¹⁰. In fact, the results of these studies differ even in the sign of the correlations obtained (perhaps because the periods studied were different, or there were biases in the data, etc.). The results of different investigations of the relationship between SST anomalies over the Indian Ocean and rainfall over India using general circulation models are also ambiguous¹¹⁻¹³. The one unequivocal result of all quantitative numerical studies is the reduction (enhancement) in precipitation directly over a cold (warm) SST anomaly.

As a first step towards unravelling the complex interaction between the continental ITCZ and the SST over monsoon regions, we investigated the relationship of the synoptic and large-scale convective cloudiness over the Indian Ocean to SST during the summer monsoon.

The basic data available for 1966-72 are: (1) daily values of a cloudiness index (ranging from 1 to 9) at each 2.5° latitude-longitude square over the tropics, based on operational nephanalysis prepared by the National Environmental Satellite Service of NOAA and compiled by Sadler and associates^{14,15}, and henceforth called the Sadler index; and (2) monthly SST over the oceanic regions between 40° E and 100° E and north of 10° S for 5° latitude-longitude squares compiled by Joseph and Pillai¹⁰ from the data collected by the voluntary observing fleet of over 40 maritime nations and stored at the National Data Centre of the India Meteorological Department.

A comparison of the daily distribution of the Sadler index with the imagery of the hemispheric mosaics analysed previously in an investigation of the variation of the ITCZ over Indian longitudes², indicated that synoptic and large-scale convection (for example, ITCZ) is associated with values of ≥ 6 of the Sadler index. Thus, we have concentrated here on epochs characterized by Sadler index ≥ 6 and define the cloudiness intensity at any grid point for any month as the sum of the Sadler index for the days on which it is ≥ 6 .

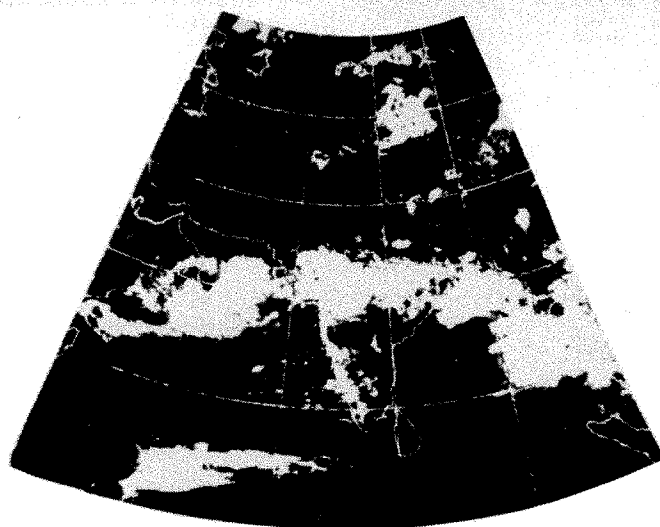


Fig. 1 The ITCZ over Indian longitudes on 8 July 1973 (after ref. 1).

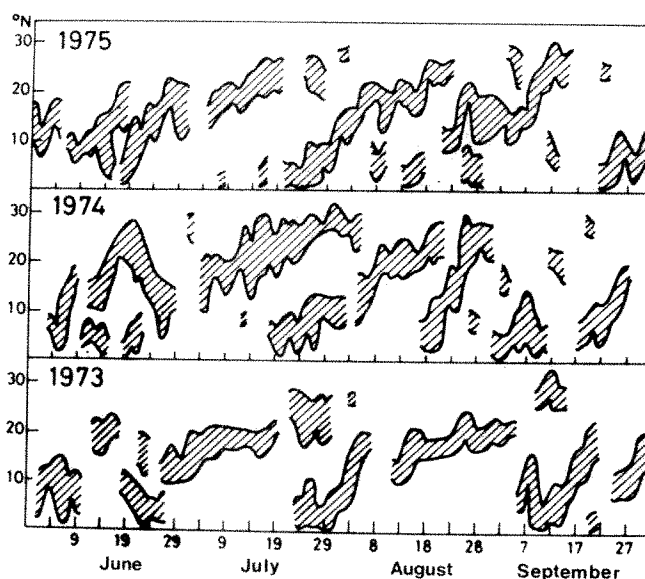


Fig. 2 Variation of the location and latitudinal extent of the ITCZ at 90° E during the monsoon seasons of 1973-75.

For a 5° grid, there are 58 squares (cells) in our study region for which SST and cloudiness intensity are given for each month (June-September) of 1966-1972. This corresponds to over 1,600 cell-months for the seven monsoon seasons. The distribution of these cell-months in the SST cloudiness intensity plane is shown in Fig. 3. The most remarkable feature of this distribution is the restriction of the points to the warmer side of the lines *ab*, *bc*. A given level of cloudiness intensity occurs only if the SST is higher than a specific value, which is clearly a necessary condition for occurrence of organized convection. For SST below 28 °C there is a well defined value for the maximum cloudiness intensity (which increases linearly with SST), whereas above 28 °C it seems to become independent of SST, although there are too few points with large cloudiness intensities to make definitive statements about this behaviour. Up to about 28 °C, the minimum cloudiness intensity is zero (that is, a Sadler index of < 6 for each day of the month) but above 28 °C it increases with SST. The probability of occurrence of different cloudiness intensities for a given SST also shows a marked change across 28 °C (Fig. 4). Whereas the most probable intensity is zero below 28 °C, it abruptly shifts to ~ 40 units above this SST. These results suggest that a SST of 28 °C is the threshold for active generation of organized convection.

Table 1 Correlation between SST and degree of cloudiness over Indian Ocean

SST greater than (°C)	No. of data points	Correlation coefficient ($\pm 95\%$ confidence limits)
24.5	1,602	0.564 ± 0.034
25.0	1,568	0.548 ± 0.035
25.5	1,502	0.517 ± 0.037
26	1,418	0.468 ± 0.041
26.5	1,340	0.418 ± 0.045
27	1,212	0.364 ± 0.050
27.5	1,064	0.282 ± 0.056
28	823	0.183 ± 0.067
28.5	468	0.094 ± 0.092
29	190	0.01

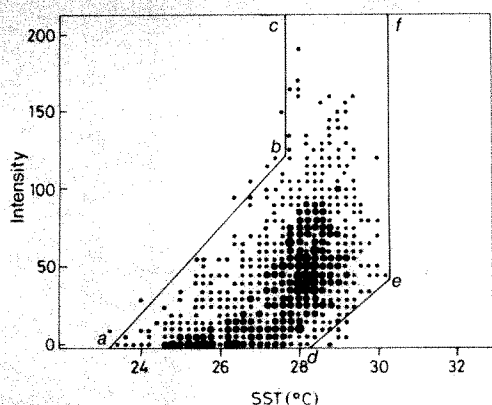


Fig. 3 Cloudiness intensity plotted against SST for June–September 1966–1972. The number of cell-months at each location is indicated: •, 1–4; ●, 5–9; ●, >10.

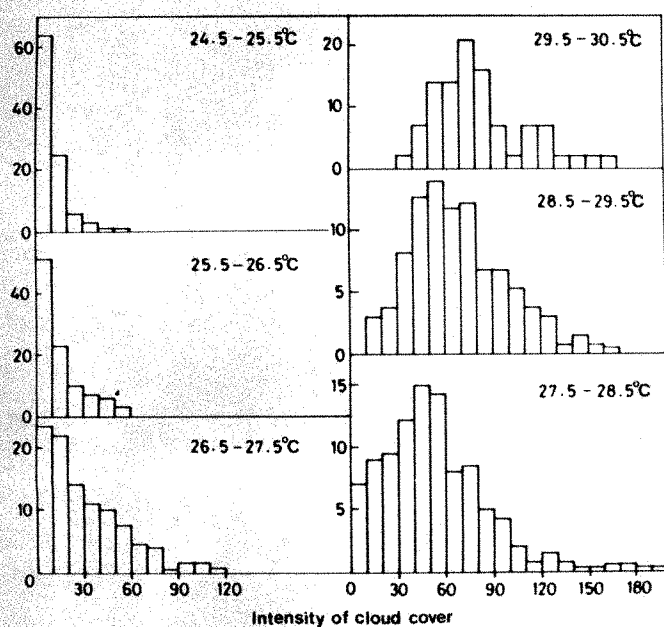


Fig. 4 The percentage of cell-months with different cloudiness intensities for SST in the given 1°C interval.

The probability of occurrence of cloudiness of 60 units (that is, about 10 'cloudy' days per month) is zero for SST below 26.5°C, which is the Palmen¹⁶ threshold for tropical cyclogenesis. This probability increases with SST, reaching a value of 0.65 (and not unity) over the warmest waters, which demonstrates clearly that high SST is a necessary but not sufficient condition for sustained cloudiness over a significant portion of the month.

The correlation between SST and cloudiness intensity based on the entire summer data set is large and positive: 0.566 ± 0.033 (95% confidence limits). Most of this correlation arises from restriction of the cloudy region to relatively warm waters, and once the threshold of 28°C is crossed, the intensity of cloud cover is no longer dependent on the SST (as seen in Table 1 for the correlation coefficient for restricted data sets comprising only cells with SST above the specified limit). Of the many factors that are important for intense convection such as lower tropospheric convergence, SST, etc., SST ceases to be the critical limiting factor when maintained above the threshold. Thus, over the warm oceans¹⁷ east of 70°E, which are the major regions of organized convection, the correlation is poor (and over some parts even negative). In fact, the observed variability in cloudiness during 1966–72 over the north Indian Ocean cannot be attributed to the variation of the monthly SST.

Received 19 March; accepted 29 August 1984.

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1,700-Myr greenstone volcanic successions in southwestern North America and isotopic evolution of Proterozoic mantle

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The formation of continental crust over the past 3,800 Myr has depleted the mantle of large-ion lithophile elements¹. A knowledge of the timing of the depletion and the degree of chemical homogeneity of the depleted mantle over the history of the Earth is important to understand the development of the continents, their recycling back into the mantle^{2–6}, and the nature of convection and mixing in the ancient mantle. This may be inferred by determining the initial $^{143}\text{Nd}/^{144}\text{Nd}$, $^{176}\text{Hf}/^{177}\text{Hf}$, and $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of igneous rocks that were derived from the mantle at various times. Initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of ~1,700-Myr greenstones from the Rocky Mountains range from $\epsilon_{\text{Nd}} = +3.3$ to $+6.5$, the highest values yet observed for mid-Proterozoic rocks. These data constrain the change in ϵ_{Nd} of depleted mantle to be only 2–3 ϵ units over the past ~1,700 Myr which supports the hypothesis of significant recycling of continental crust into the mantle^{2,3}.

Nd and Sr isotopic data were collected from ~1,700-Myr old metavolcanic rocks along a 750-km long traverse from near the Wyoming Archaean province to central New Mexico (Fig. 1). These predominantly mafic oceanic volcanic rocks probably represent primitive island-arc type magmatic effusions which are believed to be the raw materials for the generation of continental crust⁷. Lack of evidence for crust older than 1,800 Myr (ref. 8), and trace element data^{8–10} suggest that the samples are not significantly contaminated by older crust. The objectives of analysing this suite are to determine the Nd and Sr isotopic composition of the mantle 1,700 Myr ago, and to assess the meaning of the isotopic data for the evolution of the upper mantle and for the petrogenesis of these and other rocks of similar ages^{11,12}.

The volcanic greenstone successions sampled are summarized in Table 1. The U–Pb (zircon) crystallization ages for these rocks increase from south to north towards the Archaean craton (Fig. 1) (refs 13–15, 18 and S. Bowring, in preparation). The samples from the Green Mountain Formation were collected from within 3 km south of the Wyoming Shear Zone^{17–18}, which separates the ≤1,800 Myr-old crust from the >2,500 Myr Archaean province to the North.

The analytical methods are described elsewhere^{19,20}, except that neodymium isotopic measurements were made using an NdO⁺ ion beam rather than Nd⁺. Dissolution procedures are detailed in Table 1.

The Sm–Nd and Rb–Sr isotopic data are listed in Table 1. The initial ϵ_{Nd} and ϵ_{Sr} values of the samples have been calculated

Table 1 Rb-Sr and Sm-Nd isotopic data on greenstone belts, southwestern USA

Sample	Description	Sr(p.p.m.)	$^{87}\text{Rb}/^{86}\text{Sr}$	$^{87}\text{Sr}/^{86}\text{Sr}_m$	$\epsilon_{\text{Sr}}(T)^{\ddagger}$	Nd(p.p.m.)	$^{147}\text{Sm}/^{144}\text{Nd}$	$^{143}\text{Nd}/^{144}\text{Nd}_m$	$\epsilon_{\text{Nd}}(T)^*$
Tijeras greenstone ³⁴ (1,680 Myr)									
TJ-1	Tholeiite	102.7	0.5261	0.70639 $\pm$ 2	-126	4.556	0.2275	0.512463 $\pm$ 20	5.6
MZ-2	Tholeiite	137.9	0.1772	0.70402 $\pm$ 3	-39.3	4.960	0.2295	0.512525 $\pm$ 20	6.4
MZ-3	Tholeiite	132.7	0.6803	0.70855 $\pm$ 4	-148	5.893	0.2224	0.512428 $\pm$ 18	6.0
MZ-5	Tholeiite	138.4	0.0307	0.70373 $\pm$ 5	+6.9	5.234	0.2234	0.512410 $\pm$ 20	5.5
MZ-6	Tholeiite	60.40	0.0245	0.70369 $\pm$ 3	+8.2	4.490	0.2364	0.512521 $\pm$ 16	4.8
Pecos greenstone ³⁵ (1,720 Myr)									
79-12-26	High-Mg tholeiite	289.8	0.2378	0.70875 $\pm$ 3	+6.1	2.078	0.1618	0.511642 $\pm$ 15	3.9
79-22-26	High-Mg tholeiite	216.1	0.3838	0.71196 $\pm$ 3	+0.6	1.745	0.1725	0.511756 $\pm$ 19	3.8
79-18-26	Ultramafic	4.285	0.2468	0.71100 $\pm$ 4	+35.0	0.7852	0.1053	0.511154 $\pm$ 20	6.9
79-23-26	Ultramafic	4.364	0.2475	0.71096 $\pm$ 6	+34.2	0.8113	0.1056	0.511145 $\pm$ 19	6.7
		19.37	0.0444	0.70557 $\pm$ 3	+28.7	1.074	0.1364	0.511391 $\pm$ 14	4.6
Dubois greenstone ⁹ (Cachetopa Creek) (1,740 Myr)									
B-6	Tholeiite	304.5	0.0559	0.70388 $\pm$ 3	+0.7	12.69	0.1843	0.511942 $\pm$ 25	4.9
B-8	Tholeiite	226.6	0.2335	0.70750 $\pm$ 3	-11.0	11.06	0.1876	0.511995 $\pm$ 16	5.2
A-6	Tholeiite	394.0	0.0207	0.70295 $\pm$ 4	+0.1	20.69	0.1372	0.511407 $\pm$ 25	4.9
B-11	Tholeiite	464.6	0.2166	0.70772 $\pm$ 4	-1.9	27.44	0.1450	0.511476 $\pm$ 21	4.6
SAM§	Rhyolite	196.3	1.051	0.72819 $\pm$ 3	-7.6	40.82	0.1217	0.511210 $\pm$ 20	4.8
		196.4	1.057	0.72818 $\pm$ 4	-9.8	41.00	0.1217	0.511240 $\pm$ 21	5.2
Green Mountain Formation ¹⁶ (1,780 Myr)									
GM09	Tholeiite	539.1	0.0118	0.70293 $\pm$ 4	+7.1	14.50	0.1337	0.511287 $\pm$ 21	3.7
GM16	Tholeiite	464.6	0.0546	0.70411 $\pm$ 3	+4.7	16.89	0.1330	0.511308 $\pm$ 22	4.3
GM33	Tholeiite	497.3	0.0611	0.70431 $\pm$ 3	-2.7	21.47	0.1324	0.511258 $\pm$ 36	3.4
GM51	Tholeiite	606.3	0.1087	0.70605 $\pm$ 3	+12.6	19.77	0.1322	0.511266 $\pm$ 24	3.6
GM37§	Dacite	277.0	0.1297	0.72976 $\pm$ 3	+124	33.81	0.1156	0.511073 $\pm$ 21	3.7
GM27§	Rhyolite	172.8	0.6921	0.71923 $\pm$ 2	-12.4	45.64	0.1126	0.511041 $\pm$ 16	3.7
		173.0	0.6994	0.71944 $\pm$ 3	-12.1	45.68	0.1127	0.511063 $\pm$ 25	4.1
GM67§	Rhyolite	132.5	1.342	0.73652 $\pm$ 5	-3.1	30.18	0.1091	0.510995 $\pm$ 17	3.5
		134.1	1.344	0.73658 $\pm$ 3	-2.9	30.90	0.1088	0.511014 $\pm$ 26	4.1

* Calculated with respect to a chondritic reservoir with composition today of $^{143}\text{Nd}/^{144}\text{Nd} = 0.511836$, $^{147}\text{Sm}/^{144}\text{Nd} = 0.1967$, $\lambda_{\text{Sm}} = 0.00654 \times 10^{-3} \text{ Myr}^{-1}$. Error for calculated initial Nd ratio is $\pm 0.5 \epsilon$ units. The initial $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ ratios have been converted to ϵ_{Nd} and ϵ_{Sr} values, which indicate how the ratios differ from those expected for primitive mantle reservoirs at the time of formation of the lavas^{28,36}.

$$\epsilon_{\text{Nd}} = 10^4 \left[\frac{^{143}\text{Nd}/^{144}\text{Nd}_{\text{rock}}}{^{143}\text{Nd}/^{144}\text{Nd}_{\text{CHUR}}} - 1 \right]$$

† Uncertainties at the 95% confidence level. $^{143}\text{Nd}/^{144}\text{Nd}$ normalized to $^{146}\text{Nd}/^{142}\text{Nd} = 0.636151$, $^{87}\text{Sr}/^{86}\text{Sr}$ normalized to $^{86}\text{Sr}/^{88}\text{Sr} = 0.1194$.

‡ Calculated with respect to a uniform reservoir with present composition of $^{87}\text{Sr}/^{86}\text{Sr} = 0.7045$, $^{87}\text{Rb}/^{86}\text{Sr} = 0.0827$, $\lambda_{\text{Sr}} = 0.0142 \times 10^{-3} \text{ Myr}^{-1}$. Error for calculated initial Sr ratio is $\pm 0.5 \epsilon$ units for low Rb/Sr to ± 0.9 for high Rb/Sr.

§ These rhyolites were spiked and then dissolved in sealed Teflon bombs at 180 °C for 7 days. A second aliquot of each rhyolite was also completely dissolved in Teflon beakers for comparison (second entry). The small differences observed are within the range expected for sample heterogeneity (for example, see two aliquots of 79-18-26) and the calculated initial isotopic ratios are the same within analytical uncertainty.

using the zircon U-Pb ages (Table 1, Figs 2, 3). All of the initial ϵ_{Nd} values are positive. The Green Mountain Formation (GMF) has values of +3 to +4, which are comparable with those determined previously for the Twilight Gneiss and the Idaho Springs Formation orthogneiss¹¹. The ϵ_{Nd} values for the Dubois and Tijeras greenstones are in the range +4.5 to +6.5, which are the highest values yet measured on any rocks of this general age^{21,22} (Fig. 2). The basaltic rocks of the Pecos greenstones have ϵ_{Nd} values that are similar to those of the GMF. One ultramafic sample (79-18-26), however, has ϵ_{Nd} as high as +6.9, although this sample could be especially susceptible to post-crystallization disturbance because of its low Nd content. A second ultramafic sample (79-23-26) collected only 300 m from the first has a calculated initial ϵ_{Nd} of +4.5, which is more consistent with values measured for the Pecos basalts.

Because small amounts of crustal contamination would cause the ϵ_{Nd} values of magmas to be lowered, we infer that the highest ϵ_{Nd} values measured are most likely to be representative of the upper-mantle. The other rock suites may be affected by minor involvement of preexisting Archaean crust, which would have had an average ϵ_{Nd} value of about -10 (Fig. 4). The samples with the lowest ϵ_{Nd} values occur closest to the Archaean craton, which is qualitatively consistent with the contamination hypothesis. However, a wide variety of rock types from a fairly broad area all seem to have been affected to the same extent (GMF, Pecos, Idaho Springs Formation and the Twilight Gneiss). This implies that there was a rather effective and large-scale process of crust-mantle mixing involved. Only a small proportion of crust is necessary to explain the shift from $\epsilon_{\text{Nd}} = +6$ to $\epsilon_{\text{Nd}} = +4$ (1-5% depending on the process), so even the most 'contaminated' rocks still largely represent primitive mantle-derived materials.

The relatively high initial ϵ_{Nd} values of up to +6.5 (or +6.9) have important implications for crust-mantle evolution models^{3,5,6}. The relationship between these data and data on island-arc type mafic volcanic rocks of other ages is illustrated in Fig. 2. The data suggest that the ϵ_{Nd} value of the depleted mantle source of continental-type materials has increased by only about 2 or 3 ϵ units over the past 1,800 Myr. The growth of ϵ_{Nd} expected for *in situ* radioactive decay in the mantle is given by: $Q_{\text{Nd}} f_{\text{Sm}/\text{Nd}}$ (ref. 3) where Q_{Nd} is $25.13 \times 10^{-3} \text{ Myr}^{-1}$ and $f_{\text{Sm}/\text{Nd}}$ is the Sm/Nd enrichment factor relative to chondrites. The highest $f_{\text{Sm}/\text{Nd}}$ value measured on a 1,700 Myr greenstone (MZ-6) of +0.20 is similar to the highest values measured on young mid-ocean ridge basalts (MORB)²³. Based on partial melting models, the $f_{\text{Sm}/\text{Nd}}$ value of the mantle source should be larger¹. Consequently, the growth rate of ϵ_{Nd} in the mantle due to *in situ* radioactive decay is expected to be $> 5 \times 10^{-3} \text{ Myr}^{-1}$, or ~3-5 times faster than observed. These data verify the discrepancy noted previously³ and emphasize the need to have low- ϵ_{Nd} materials injected into the mantle, to slow the growth of the upper mantle ϵ_{Nd} value. We believe that this low- ϵ_{Nd} material is old continental crust, although other explanations have been proposed²⁴. The data also constrain the depleted mantle curve for mid-Proterozoic time to significantly higher ϵ_{Nd} values than previously suggested^{11,25} (Fig. 2).

Poor correlation of measured $^{87}\text{Rb}/^{86}\text{Sr}$ with $^{87}\text{Sr}/^{86}\text{Sr}$ (Table 1) indicates disturbance of the Rb-Sr systematics of the samples. This casts doubt on their reliability as estimators of the mantle $^{87}\text{Sr}/^{86}\text{Sr}$ value. The calculated initial values for the samples are compared with present-day oceanic basalt values on the $\epsilon_{\text{Nd}} - \epsilon_{\text{Sr}}$ diagram^{30,31} (Fig. 3). This representation assumes that the present-day whole Earth $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{87}\text{Rb}/^{86}\text{Sr}$ ratios are 0.7045 and 0.0827 respectively²⁶. The ϵ_{Sr} values for the Proterozoic

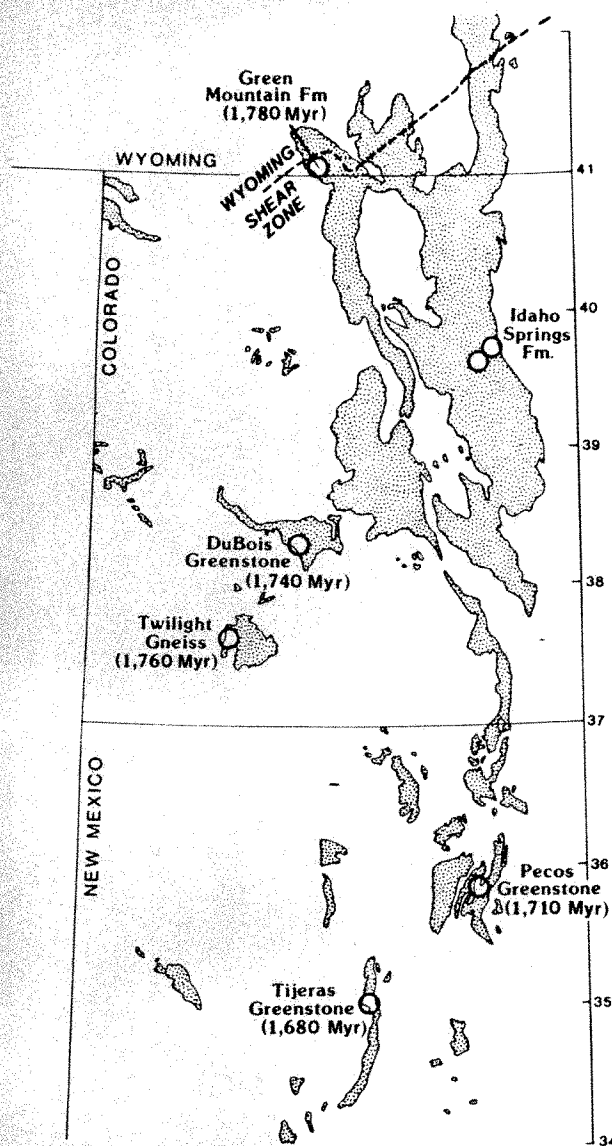


Fig. 1 Precambrian outcrop map of Wyoming, Colorado and New Mexico, USA showing sample locations and U-Pb ages from zircons (adapted from ref. 8).

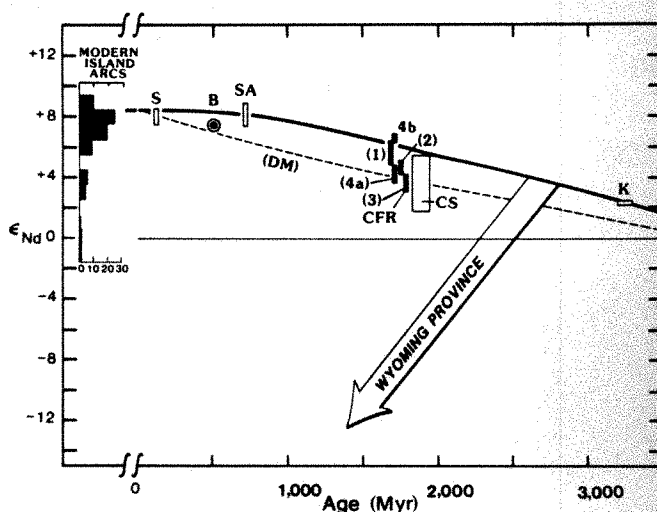


Fig. 2 Nd isotopic evolution diagram for the depleted mantle. Data from this work are shown in solid rectangles. (1), Tijeras; (2), Dubois; (3), Green Mountain Formation, which corresponds to the range for the Colorado Front Range¹¹ (CFR), (4a) Pecos, (4b) possibly altered ultramafic sample from Pecos. Large arrow indicates average evolution for Archaean crust^{37,38}. Other isotopic data: K, Kambalda³⁹; CS, Cape Smith²¹; SA, Saudi Arabia⁴⁰; B, Bay of Islands ophiolite²⁹; S, Samail ophiolite⁴¹. A histogram of published ϵ_{Nd} values for modern intra-oceanic island arcs apparently free from contamination by older crust is shown at left^{28,42-50}. All samples with $\epsilon_{Nd} < 6.5$ are either boninites or from the Lesser Antilles. Dashed curve (labelled DM) is isotopic evolution of depleted mantle proposed by DePaolo¹¹ and Allègre and Rousseau²⁵.

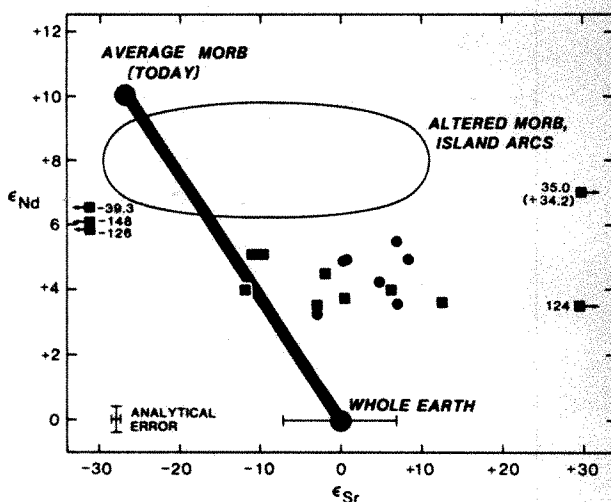


Fig. 3 ϵ_{Nd} versus ϵ_{Sr} diagram, showing initial ratios calculated for samples with $^{87}Rb/^{86}Sr < 0.1$ (●) and $^{87}Rb/^{86}Sr > 0.1$ (■). (Data that plot outside range of graph are indicated by arrows with ϵ_{Sr} values.) Oceanic correlation line for modern basalts corresponds to $\epsilon_{Sr} = -2.7\epsilon_{Nd}$. Value and estimated errors for bulk Earth isotopic composition are from ref. 26. Depleted mantle should always have values along the correlation line (see text). Three samples plot near this line, but typically the Rb-Sr systems have been strongly disturbed by later metamorphic or weathering events.

metabasalts with $^{87}Rb/^{86}Sr < 0.1$ appear to be too high relative to their ϵ_{Nd} values when compared with young oceanic lavas, especially those from mid-ocean ridges^{23,26,27}. All except three of the samples plot to the right of the line $\epsilon_{Sr} = -2.7\epsilon_{Nd}$. The three anomalous samples have impossibly low calculated initial ϵ_{Sr} values and are clearly disturbed. Three of the samples with higher $^{87}Rb/^{86}Sr$ ratios lie close to the line. The apparent shift of ϵ_{Sr} values to the right on the diagram is similar to altered young oceanic basalts and some island-arc lavas²⁶⁻²⁹. This, and the relatively large range of observed ϵ_{Sr} values lead us to conclude that the samples do not precisely define the ϵ_{Sr} value of the mantle sources of the lavas. Most simple models of mantle isotopic evolution predict that the ϵ_{Sr} value of depleted mantle reservoirs should lie near the MORB-whole Earth line for all time. This follows from the observation that the partitioning of Sr and Nd between the mantle and the continental crust may be similar³⁰, though this has not been proven. ϵ_{Nd} values of +6 should correspond to $\epsilon_{Sr} = -16$ or $^{87}Sr/^{86}Sr \approx 0.7014$ at ~1,700 Myr ago. Most previous studies of mid-Proterozoic rocks have failed to find initial $^{87}Rb/^{86}Sr$ values this low^{21,31}. Our data are consistent with this low value for the mantle, but cannot be considered proof because of the uncertainties associated with

the effects of metamorphism. We emphasize that the observed initial $^{87}Rb/^{86}Sr$ ratios in the high-Rb/Sr rocks (as low as 0.7023) would normally be considered to be quite low, but the correct mantle value is probably lower.

Greenstones of 1,680–1,780 Myr age in the southern Rocky Mountains have high ϵ_{Nd} values and high Sm/Nd ratios that indicate the existence of relatively homogenous and widespread depleted mantle reservoirs similar to those sampled today in oceanic magmatic arcs. The data strengthen the observation³

that the growth of ε_{Nd} in the upper mantle has been retarded over the past 2,000 Myr, probably because of recycling of continental crust. The general tendency for samples nearer the Archaean craton to have lower initial ε_{Nd} values argues for contamination with small amounts of Archaean crust. Yet these samples—the GMF, Twilight Gneiss and Idaho Springs Formation—all have the same calculated initial $\varepsilon_{Nd} = 3.7 \pm 0.5$, which implies vigorous and thorough regional mixing of a small amount of crust into the mantle near the Archaean plate boundary. The almost ubiquitous disturbance of the Rb-Sr system in crustal rocks makes it difficult to determine a precise $^{87}Sr/^{86}Sr$ evolution curve for depleted mantle. Continuing efforts to establish this curve are, however, extremely important in the context of establishing the history of crust-mantle evolution.

The Nd data confirm earlier conclusions that large segments of the crust in southwestern North America were derived from the mantle ~1,800 Myr ago³². Compared with the Mesozoic and Cenozoic patterns, this suggests major contrasts in rates, and possibly styles, of plate tectonics in middle Proterozoic time³³.

We thank Kent Condie and James Robertson for providing well documented samples, Sam Bowring for discussions of regional geology, and P. J. Patchett for reviewing the manuscript. This work was supported by a grant from donors of the Petroleum Research Fund, administered by the American Chemical Society, and NSF grant EAR82-12377.

Received 26 April; accepted 15 August 1984.

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A mechanism for magmatic accretion under spreading centres

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The most active volcanic zone on Earth occurs along the global mid-ocean ridge system where the lithosphere is pulled apart and new oceanic crust is created at rates between 10 and 200 km Myr⁻¹. This volcanism is segmented, and is concentrated along the axial valleys and highs of slow- and fast-spreading ridges respectively, with transform fault zones separating, and generally offsetting, each active ridge segment. Volcanic activity may be nearly absent at the ridge transform intersection where a slow-spreading ridge is offset, and greatly reduced where a fast-spreading ridge is offset, implying thinner crust in these regions¹. The source of magmas erupted along ocean ridges is the underlying mantle, which undergoes decompression melting when rising to fill the gap between the spreading plates. The resulting magma aggregates in the upper mantle and ascends to the crust due to its low density compared with the parent mantle rock. The melt is believed to pool in crustal magma chambers—whence it periodically erupts to the surface. Typically, the formation, ascent and aggregation of magmas along the mid-ocean ridges is regarded two-dimensionally and modelled in a section through the crust and mantle across the strike of the ridges (see ref. 2). Here, however, we consider the problem with a three-dimensional model for which we provide supporting geological evidence, in which the magmas rise as a result of a gravitational instability (modified Rayleigh-Taylor) in the underlying partial melt zone.

It is reasonable, to a first approximation, to assume that the mantle is behaving as a viscous newtonian fluid, so we can expect the upwelling to be two dimensional with a maximum under the smoothed location of the spreading centre. If this is the case, we also postulate that a linear region of high-melt content exists in the mantle below the ridge where melt aggregates from the rising asthenosphere. This region could be approximated as a horizontal cylindrical body with relatively low viscosity and density compared with the overlying mantle. In such circumstances, it will develop a gravitational instability leading to regularly spaced vertical protrusions. We have conducted some simple experiments in which a water-glycerine mixture was quickly injected into glycerine along a horizontal line. Although this line will gradually rise because the water-glycerine mixture is less dense than the pure glycerine, an instability will always also develop as shown in Fig. 1 and lead to semi-spherical pockets. It is reasonable to expect that a linear

Table 1 Composition of postulated parental mantle, abyssal peridotites and a primitive abyssal basalt

	Parental mantle models			Fracture zone peridotites§	Primitive abyssal basalt tholeiite
	Pyrolite*	Pyrolite†	Xenoliths‡		
SiO ₂	46.1	45.0	42.9	43.6	50.9
TiO ₂	0.02	0.17	0.2	0.02	0.89
Al ₂ O ₃	4.3	4.4	5.8	1.18	16.0
FeO	8.2	7.6	9.2	8.20	8.83
MgO	37.6	38.8	37.2	45.2	9.02
CaO	3.1	3.4	3.7	1.13	12.4
Na ₂ O	0.04	0.40	0.7	0.02	2.08
Cr ₂ O ₃	—	0.45	0.2	0.22	—
NiO	—	0.26	0.2	0.22	—
Mg/(Mg + Fe)	0.891	0.901	0.878	0.908	—

* Pyrolite model²¹.

† Pyrolite model²².

‡ Upper mantle model²³.

§ Average abyssal peridotite⁹.

|| Primitive abyssal tholeiite (TR154-1802)²⁴.

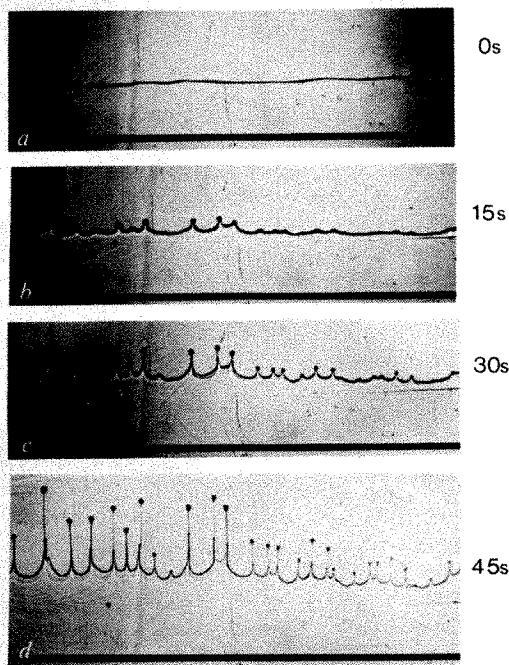


Fig. 1 Gravitational instability of a horizontal line of water/glycerine mixture in a bath of pure glycerine. Kinematic viscosity of the glycerine is estimated as $5.0 \text{ cm}^2 \text{ s}^{-1}$, that of the water/glycerine mixture as $0.015 \text{ cm}^2 \text{ s}^{-1}$, and reduced gravity $g^* = g\Delta\rho/\rho = 260 \text{ cm s}^{-2}$. The dark stripe at the bottom is 0.64 cm diameter tubing, and the initial diameter of the conduit is estimated with a precision optical micrometer to range from 0.16 to 0.24 cm. The spacing of the 25 diapirs in *d* is 39.5 cm for an average of 1.6-cm spacing, giving a ratio of spacing to conduit diameter of 7–10. Marsh argues that the formula for spacing is $d/D = 2\pi/2.15 (\mu_1/\mu_2)^{1/3}$, where μ_1 is viscosity of the upper fluid and μ_2 is viscosity of the conduit fluid with perhaps an order one correction in the coefficient⁴. Our parameter group $2\pi/2.15 (\mu_1/\mu_2)^{1/3}$ is ~ 20 , roughly twice as big, but well within the acceptable range given.

region of partially molten mantle in the Earth will behave in a similar manner and will lead to fairly regularly spaced protrusions from which the melt will ascend to form magma chambers at spreading ridges.

The instability is similar to the classical Rayleigh–Taylor instability in fluid mechanics. It occurs when there is a density inversion, that is a layer of heavy fluid over light. Whitehead and Luther³ reported some experiments and theory on this instability which were relevant when both viscosities were large. They found that the ratio of the wavelength of fastest-growing disturbances to the thickness of the unstable layer was large as the viscosity contrast became large. Thus, a thin layer of relatively low-viscosity fluid can be expected to develop very widely spaced protrusions. Marsh^{4,5} isolated a similar problem, the instability of a ribbon-like, or cylindrical (with its axis horizontal), body of low-viscosity material. The ribbon-like region was produced in the laboratory by allowing black oil or glycerine to rise from a slit in the bottom of a container of glycerine. Marsh suggests that this diapir-genesis occurs under island arcs and is the mechanism that leads to the formation of the magma chambers for the island-arc volcanoes. Noting the regular spacing of magmatic centres across Iceland, Sigurdsson and Sparks⁶ suggest that a similar instability exists at the base of the lithosphere in Iceland.

The suggestion that there is convection at right angles to the direction of relative plate movement is not new (see ref. 7). However, we suggest that an instability due to density change through melting in the mantle beneath the ridge produces upwellings with length scales of the spacing between the midpoints of spreading centre segments. This also implies that axial magma chambers are localized at the midpoints of individual ridge segments.

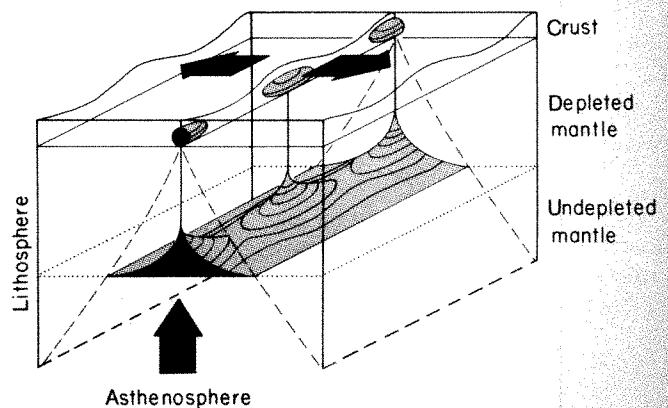


Fig. 2 Viscous asthenosphere rises two-dimensionally beneath the boundary between two spreading lithospheric plates. The lithosphere thickens away from the spreading boundary (dashed lines). Above a certain level (dotted lines), the rising asthenosphere passes through a zone in which partial melt can form which collects at some level below the base of the lithosphere. Due to its lower viscosity and density, the partial-melt zone develops a gravitational instability leading to regularly spaced concentrations of melt which rise to the surface to form crustal magma chambers. The asthenosphere, effectively depleted by this process, must continue to rise viscously and on cooling become lithosphere. The wavelength of the gravitational instability (and the subsequent spacing of spreading centres or transform zones) should also depend on the width of the partial melt zone which is expected to be proportional to the spreading rate.

Evidence for a partially molten zone beneath the base of the lithosphere is seen from regional magnetotelluric measurements in Iceland. These measurements indicate an anomalous conducting layer at a depth of 8–15 km beneath the Neovolcanic Zone which is interpreted as a partially molten zone near the top of the mantle⁸. This zone may correspond to the partially molten region we require in the mantle beneath mid-ocean ridges.

Direct evidence for melt accumulation from a partially molten zone beneath the mid-ocean ridges can be seen in plagioclase-bearing peridotites from oceanic fracture zones. These rocks constitute $\sim 30\%$ of dredged abyssal peridotites⁹ and generally contain $<0.5\%$ interstitial plagioclase which is believed to have largely crystallized from trapped melt^{10–12}. Given the proportion of plagioclase in primitive abyssal tholeiite, it represents $<1\%$ trapped melt in the peridotites, although in rare cases, as locally along the Romanche Fracture Zone, peridotites from one or more dredge haul contain an average of $\geq 6\%$ plagioclase⁹ corresponding to $\sim 13\%$ trapped melt. This is direct evidence for local accumulation of melt in the mantle, which we believe is the result of a Rayleigh–Taylor instability in the partially molten zone and a rapid migration of melt towards the resulting protrusions into the overlying mantle. Green and Hibberson¹³ studied the stability of plagioclase in peridotites at high pressures and found that it was unstable at pressures >8.5 kbar. Based on these phase relations, the zone of melt aggregation represented by the plagioclase-peridotites must lie at <25 km in the mantle.

Abyssal peridotites dredged largely from oceanic fracture zones are generally accepted as the residues of mantle melting which generated abyssal basalts. Almost 70% of these peridotites are plagioclase-free spinel peridotites⁹. Table 1 compares their average composition with independent estimates of undepleted upper mantle composition and a typical primitive abyssal basalt. This shows that melting of the upper mantle would rapidly deplete the solid residue in those elements which occur in far greater abundance in basalt than in the postulated source rock. From the relative concentrations of CaO and Al_2O_3 in Table 1, somewhere between 15 and 20% melt would have to be removed from the postulated parents to produce the observed concentrations of these oxides in the fracture zone peridotites.

Taking all abyssal peridotites together gives an average of 0.9% plagioclase which would correspond to ~1–2% trapped melt. Thus most of the melt which was formed in peridotites dredged from fracture zones must have been drained off. Most of these rocks, however, were dredged from regions in fracture zones where only peridotites were exposed for areas of hundreds, if not thousands, of square kilometres and where little, if any, basalt exists. Accordingly, the melts were not emplaced in the same region as their parental peridotites. We propose that melt formed at depth near fracture zones migrates laterally through the mantle towards an upwelling beneath the centre of the ridge segment rather than that the depleted peridotites were emplaced laterally into the fracture zone after melting beneath the ridge.

Figure 2 summarizes our suggested structure and flow field. The asthenosphere rises viscously beneath the spreading centres. Above a certain level, the rising asthenosphere enters a zone in which partial melt can form. To a first approximation the viscous flow of the asthenosphere is two-dimensional with its axis parallel to the ridge. Thus the rate of partial melt formation is nearly uniform below the ridge axis. The partially molten zone will have relatively low viscosity and density compared with the overlying mantle. The partially molten zone thus develops regularly spaced protrusions centred beneath individual ridge segments. The melt, which can migrate by porous flow, may move more rapidly than the partially molten layer as a whole and concentrate at the tops of the rising protrusions. This would lead to gravitational separation of melt from the mantle and its ascent to form or feed crustal magma chambers. Above the melt aggregation zone, the depleted asthenosphere must continue to rise viscously and, on cooling, become lithosphere.

Young melt concentrations will replenish the older crustal magma chambers. New crust is formed by crystallization of gabbro along the margins of the magma chambers, by the injection of dykes from the magma chambers into the existing crust, both laterally and vertically along the spreading centre axes, and by extrusion of pillow basalts and massive flows from magma chambers and dykes to the top of the crust. Furthest away from the crustal magma chambers, that is, in the transform zones between spreading centres, fewer dykes will be injected and accordingly fewer pillow basalts and massive flows will be extruded, thus producing thinner crust.

This model provides an explanation for the regular segmentation of ocean ridges. Schouten and Klitgord¹⁴ postulated that the sea floor in the slow-spreading North Atlantic (and in other oceans) was formed in a relatively stationary series of spreading centre cells separated by transform-fault zones. From Mesozoic marine magnetic anomalies (155–108 Myr ago) in the western North Atlantic, they estimate a typical separation between spreading centre cells of the order of 50 km, similar to the present distribution of axial valleys on the Mid-Atlantic Ridge. The magnitude of offset between adjacent spreading centres is typically <30 km, can change with time, and can even go through zero and change sign¹⁵. This is inconsistent with an offset-generating mechanism inferred from the rheology of the plate material alone, as the ridges should lose their 'memory' of the fracture zone at 'zero offset'. The stability of spreading centre cells, then appears independent of offsets between them^{14–16}. The model proposed here predicts regular segmentation of ocean ridges, particularly where no large-offset transforms exist. The model also predicts that the crust at small and zero-offset fracture zones should thin, which is consistent with what little is known from seismic studies of these features^{15,17–19}.

Lateral flow of melt towards regularly-spaced protrusions centred beneath individual ridge segments would inhibit flow and mixing of melt in the mantle between ridge segments. Accordingly, melts erupted at each ridge segment will be derived largely from the mantle beneath that segment. This provides a simple explanation for the geochemical discontinuities observed in ridge basalts across fracture zones (see ref. 20) and strongly supports a laterally heterogeneous oceanic mantle. This effect is heightened at large-offset fracture zones where old-cold lithosphere, juxtaposed against the rising asthenosphere, depresses

the partially molten zone¹ and produces a substantial physical barrier to the lateral flow of magmas. We note that our model does not rule out segregation and intrusion of melt to the crust from greater depths in the mantle, nor does it rule out the occurrence of secondary instabilities in the partially molten layer either near fracture zones or laterally away from the ridge axis. We point out, however, that it does predict the presence of geochemical discontinuities in ridge basalts even at zero-offset fracture zones.

This paper developed from a series of interdisciplinary seminars and direct research support sponsored by the Center for the Analysis of Marine Systems of the Woods Hole Oceanographic Institution with funds provided by the Exxon Education Foundation. We thank B. D. Marsh, W. B. Bryan, S. Humphries and J. Karson for constructive criticism. We acknowledge support from the NSF and the Office of Naval Research. Robert Frazel aided in design and construction of the experiment and photography. Anne-Marie Michael, Alice Tricca and Pam Foster processed the manuscript. Woods Hole Oceanographic Institution contribution no. 5668.

Received 2 May; accepted 14 August 1984.

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Anaerobic consumption of organic matter in modern marine sediments

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Previous estimates of the global organic carbon (C_{org}) budget have generally considered the anaerobic consumption of C_{org} to be of minor importance. Data for the rates of bacterial sulphate reduction in various morphological zones of the oceans indicate, however, that approximately 14% of the total C_{org} reaching the sediment-water interface is consumed by anaerobic processes. Of this, some 70% is taken up in sediments on the continental shelves and slopes in water depths of less than 1,000 m. Although this represents only a small fraction of the global primary carbon productivity, it may have important consequences for models of the carbon and sulphur cycles.

There is some controversy over the rate of primary productivity in the world ocean. Values ranging from 20 Gton $C yr^{-1}$ (refs 1, 2) to 126 Gton $C yr^{-1}$ (ref. 3) have been reported. In view of recent work which questions the validity of the ^{14}C -technique⁴ and considers contributions from picoplank-

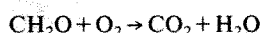
Table 1 C_{org} budget for the top 1 m section of shelf sediments off Mexico (Station 668, depth 140 m, 23°23'N, 106°56'9W)

	C_{org} flux
Primary production in the region	$2.5 \text{ g m}^{-2} \text{ d}^{-1}$ ($= 0.91 \text{ kg m}^{-2} \text{ yr}^{-1}$)
C_{org} flux to sea floor (15% of primary production) ¹⁰	$0.14 \text{ kg m}^{-2} \text{ yr}^{-1}$
C_{org} consumption for bacterial sulphate reduction at the rate of $55.7 \mu\text{g S per kg sediment per day}$ (ref. 16)	$41.8 \mu\text{g C per kg sediment per day}$ $22.9 \text{ g C m}^{-2} \text{ yr}^{-1}$ ($= 229 \text{ kg C m}^{-2} \text{ per } 10^4 \text{ yr}$)
Thus, C_{org} consumption for anaerobic bacterial sulphate reduction	
Residual C_{org} (top 1 m) at 3% C_{org} and 60% water	18 kg C m^{-2}
C_{org} consumption for aerobic respiration for the top 1 m at O_2 consumption of $10 \text{ mol m}^{-2} \text{ d}^{-1}$	$1,168 \text{ kg C per m}^{-2} \text{ per } 10^4 \text{ yr}$
Hence the total C_{org} consumption in the uppermost metre of sediment ($229 + 18 + 1,168$) = $1,415 \text{ kg C m}^{-2} \text{ per } 10^4 \text{ yr}$.	

The rate of sedimentation is 10 cm kyr^{-1} (ref. 24), thus 1 m is assumed to have been deposited in 10^4 yr . The wet density is 1.5 g cm^{-3} and this is therefore equivalent to $1,500 \text{ kg m}^{-2}$.

ton^{5,6}, chemo- autotrophic⁷ and lithotrophic bacteria⁸, Riley's³ higher value seems the more realistic. Mineralization in the euphotic zone by grazers or chemical oxidation recycles ~60–80% of the particulate organic matter (POM) and, depending on water depth, all or none of its will reach the sea floor.

The POM which ultimately reaches the sediment–water interface will partly be remineralized in the benthic layer⁹ or through the action of burrowing organisms and microbes. It has been estimated that 527,109 moles O_2 are consumed daily in marine sediments¹⁰. This allows POM consumption figures for aerobic processes in bottom sediments to be derived assuming the reaction



to be a reasonable approximation. This yields a value for POM consumption of $2,308 \text{ Gton C yr}^{-1}$.

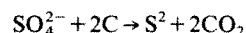
However, anaerobic processes are also at work in marine sediments and I have attempted to estimate the anaerobic C_{org} remineralization in recent marine sediments.

One way of calculating the anaerobic remineralization of POM is to use the difference in C_{org} or N_{org} values between the top and bottom sections of piston cores and take the resulting decay rate for the two compounds as a measure of anaerobic respiration of POM¹¹. This method, however, has great limitations because even the top sediment layers retrieved in the core material may already contain some biologically inert POM and furthermore, rates of sedimentation may vary considerably between the top and the bottom of the core.

Another approach, which utilizes C_{org} consumption rates, is based on the reduction of manganese, iron and sulphur compounds; estimates of respiration are obtained according to a formula developed by Upsensky and Strakhova^{12–15}. In principle, C_{org} consumption is related to the generation of certain solid phases such as pyrite, but the POM consumed when other oxidized species are reduced, is neglected. Strakhov¹⁵ was the first to draw attention to this oversight. He pointed out that additional factors control POM utilization in marine sediments, such as the release of COOH , CH_4 or NH_3 .

Recent biogeochemical findings suggest that not all H_2S generated by reduction of sulphate will enter a solid phase. Some H_2S will migrate upwards or become oxidized. Figure 1 depicts the relationships between various oxidized and reduced sulphur species encountered in representative sediment cores from the Pacific^{15,16}. Sulphate in interstitial water is highly enriched in ^{32}S , with $\delta^{34}\text{S}$ values in the range of +15.6 to +18.8‰, compared with a $\delta^{34}\text{S}$ value of +20‰ for seawater sulphate. This indicates that the sub-surface sulphate maximum (Fig. 1) is caused by the oxidation of isotopically-light metabolic H_2S rather than by a conservative uptake of seawater sulphate during deposition. Analysis of all three forms of sulphur, that is gaseous, dissolved and solid, from anaerobic sediments of Limfjord, Denmark¹⁷ reveals that only 10% of the H_2S generated *in situ* is associated with sulphides and disulphides. The remaining 90% diffuses from shallow muds to near bottom water or on oxidation becomes part of the general sulphur cycle in sediments. From these few but representative examples, it is inferred that reduced sediments, especially those generating free H_2S remineralize more C_{org} during anaerobic microbial early diagenesis than would be expected from the amount of pyrite or other sulphur-containing minerals that are present. These apparent shortcomings can be overcome by using radioactive sulphur- and carbon-isotope techniques to trace and quantify C_{org} remineralization in anoxic sediments^{16–18}.

Bacterial sulphate reduction accounts for 90–95% of the anaerobic remineralized POM^{19,20}. Thus to estimate C_{org} consumption in conditions of anaerobic diagenesis it is sufficient to know the rate at which bacterial sulphate is reduced in sediments. Sulphate reduction can briefly be summarized as follows:



(that is 1 mole S oxidizes 2 moles C) which is a quantitative expression of anaerobic remineralization.

Quantitative data on sulphate reduction in the open sea, in marine sediments, and that obtained in laboratory conditions using radioactive isotope tracer techniques agree very well. Therefore, it is possible to calculate rates of microbial activities for the top metre of modern oceanic sediments and to evaluate C_{org} consumption in conditions of early diagenesis in anoxic media^{11,17,19–21}. In a few instances, knowledge of sedimentation

Table 2 Estimates of global bacterial sulphate reduction in the uppermost metre of oceanic sediments

Geomorphological zone	Depth (m)	Area (10^6 km^2)	Intensity of sulphate reduction ($\mu\text{g S kg}^{-1} \text{ d}^{-1}$) (mg S $\text{kg}^{-1} \text{ yr}^{-1}$)		Productivity of sulphate reduction (g S $\text{m}^{-2} \text{ yr}^{-1}$)	Flux of $\Sigma \text{H}_2\text{S}$ ($10^6 \text{ tons yr}^{-1}$)	C_{org} consumption for sulphate reduction ($10^6 \text{ tons C yr}^{-1}$)	(%)
Shelf	0–50	11(2)	55.6	20.3	26.4	79.2	59.5	16
	50–200	16(3)	37.3	13.6	17.7	53.1	40.0	11
Continental slope	200–1,000	(15)	28.2	10.3	13.4	201.0	151.0	41
	1,000–3,000	(61)	5.5	2.0	2.6	158.6	119.2	32
Foot of slopes, depressions, oceanic bed	>3,000	257	0.97	0.35	—	—	—	—
Total		360 (81)				491.9	369.7	100

Estimates are after refs 16, 18, 20, 21, 25, 26. The figures in parentheses give the areas of biogeochemically active sediments with bacterial sulphate reduction.

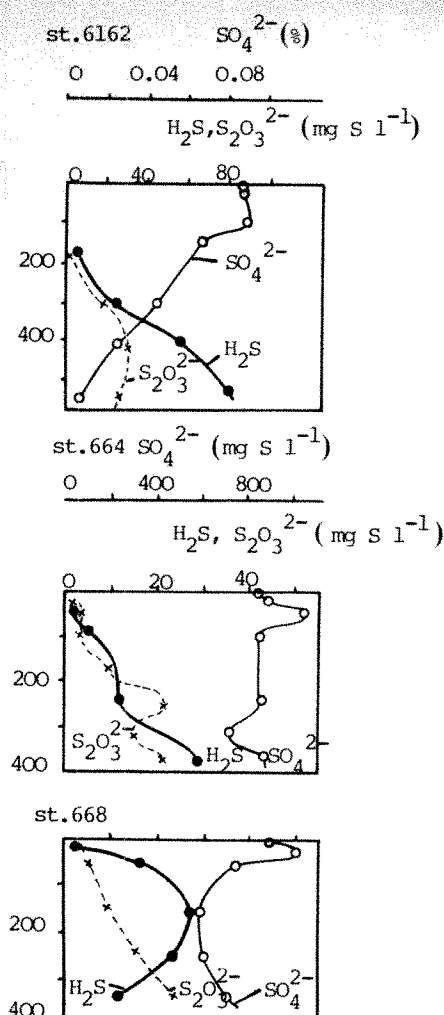


Fig. 1 Depth distribution (in cm) of dissolved sulphur species in pore waters of anoxic oceanic sediments. For location of stations and further details see ref. 24.

rates and the rate of bacterial sulphate reduction (from ^{35}S tracer techniques) allows the calculation of C_{org} budgets. Table 1 summarizes the data for shelf sediments off Mexico. In brief, $\sim 1,400 \text{ kg } C_{\text{org}} \text{ per m}^2 \text{ per } 10^4 \text{ yr}$ enter the marine sediments, of which $<20\%$ are consumed by anaerobic processes. There is a close correspondence between the estimates of C_{org} flux to the sea floor and the total C_{org} consumption during diagenesis.

Experimental data from about 30 stations are compiled in Table 2. This data set covers all critical morphometric zones of the world ocean^{16,17,20,21} and reveals mean global reduction rates for the upper 1 m of the surface sediment layer. About 70% of the total C_{org} consumed anaerobically occurs in sediments down to water depths of 1,000 m. The annual production of metabolic H_2S per square metre sediment of sediment and C_{org} consumed for this process were calculated from the data on the reduction intensity. C_{org} consumption for anaerobic bacterial processes in diagenesis makes up nearly 14% of the total C_{org} arriving at the world ocean bottom (Table 3).

Table 3 Global oceanic C_{org} budget

Process	$10^6 \text{ tons yr}^{-1}$	%
C_{org} consumption:		
aerobic ¹⁰	2,308	83
anaerobic	370	14
C_{org} buried ¹⁶	85	3
Total C_{org} flux to the bottom	2,763	100

Although the above conclusions are reached using comparatively few data points, the global validity of the suggested approach appears to be more meaningful and realistic than pathways taken by previous investigators^{11-15,22,23}. This is because not only solid mineral phases but also a wide variety of gaseous and soluble metabolic products have been considered in the present quantification of anaerobic bacterial processes operating in modern marine sediments.

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A 7,272-year tree-ring chronology for western Europe

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Long tree-ring chronologies provide a unique calendrical record that is of value for archaeological dating, climatic and post-glacial studies. They also form a standard for the calibration of the radiocarbon time scale. The world's longest continuous tree-ring chronology is based on the bristlecone pine (*Pinus aristata* and *Pinus longaeva*) growing in the White Mountains of California¹⁻³. The great age of living and sub-fossil trees of this species enabled a continuous tree-ring sequence of 8,681 years to be established, providing absolutely dated wood samples for the first radiocarbon calibration^{4,5}. We have now established an unbroken west European tree-ring sequence spanning the past 7,272 years.

Figure 1 shows the areas from which the samples are derived. The deciduous oaks (*Quercus petraea* and *Quercus robur*) used for both the German and Irish tree-ring studies show several marked differences from the bristlecone pines. They all grew at low altitude ($<200 \text{ m}$ in Ireland and $<400 \text{ m}$ in Germany, compared with $\sim 3,000 \text{ m}$ for the bristlecone pines), with average life spans of only 150-250 yr compared with 1,000 yr or more for bristlecone pines. Hence, the establishment of this long oak chronology required the linking together of a great number of tree-ring records by the process of cross-dating.

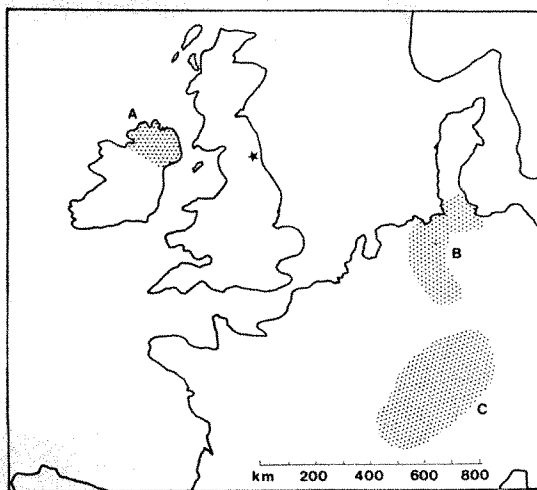


Fig. 1 Map to show the origin of oaks that contributed to the European oak chronology. Area A (north of Ireland), mostly bog oaks; area B (northern Germany), bog oaks and archaeological timbers; area C, southern Germany, mostly river valley oaks. The star indicates the site of Swan Carr in eastern England.

The synchronism of sequences of tree-ring widths is determined by visual and statistical comparison of the patterns of wide and narrow rings. The average growth increment for each year, derived from a number of trees, forms the tree-ring chronology. In both Ireland and Germany, individual ring patterns were cross-dated and merged to form site chronologies. The chronology building process consisted of linking those internally consistent site chronologies, typically of 500–1,000 yr length, to form even longer floating chronologies^{6–8}. Eventually these long floating chronologies were linked to the sequences from living trees to form an absolute time scale. By 1982, a continuous sequence existed in Germany extending back 4,000 yr⁸ (recently extended to 6,000 yr⁹), and in Ireland there was a potential sequence extending back 7,200 yr from the present, with only a single gap between 200 and 13 BC and 13 BC¹⁰.

There is no theoretical basis for judging the geographical distance over which cross-dating will be feasible, so attempts to cross-date the Irish and German sequences were based on previous empirical experience. Successful stepwise cross-dating has been achieved in a west-east transect from eastern France to eastern Austria¹¹, in a north-south direction from Swiss lake dwellings to the south German valleys^{12–14} and up to the southern coastal area of the Baltic Sea^{8,15,16}. It has also been shown that cross-dating is possible from Ireland to Germany by stepwise comparison of chronologies from Ireland to England to Germany^{17,18}. For example, in the modern era, sections of English chronology can be dated to the same calendar year against both the independent Irish and German chronologies. In attempting to establish cross-dating directly between Irish and

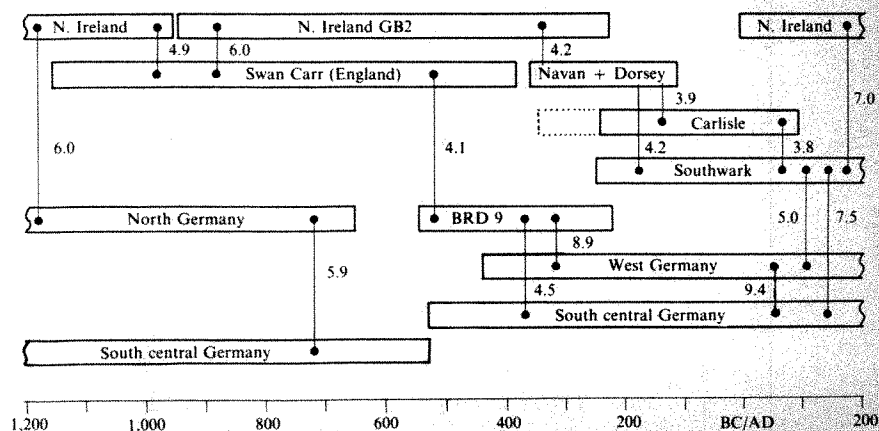
German oak chronologies, one must bear in mind the differences in climatic and ecological conditions. For example, the Irish and north German bog oaks had a different hydrological environment from the river valley oaks of southern Germany and from the dry land oaks used in archaeological sites. However, in spite of these ecological differences and the considerable distances involved, the statistical significance and consistency of the cross-dating is sufficient to ensure that both chronologies are exactly synchronized.

Reliable cross-dating between the north of Ireland and northern Germany for the second millennium BC was established in early 1982 and appeared to place a long floating section of Irish chronology into the exact time span 5218–158 BC. However, by mid-1982, work on timbers from the Roman site at Carlisle in northern England and from Iron Age sites at Navan and Dorsey in Northern Ireland had established a tentative link between the absolute chronologies of the AD era and the young end of the Irish sub-fossil chronology. This specified the end date of the floating Irish sequence as 229 BC instead of 158 BC, as suggested by the link with German chronologies. Baillie¹⁰ pointed out that the dating of the entire German chronology complex earlier than 550 BC was dependent on timbers from a single site, Kirnsulzbach, dated by Hollstein¹⁹. The chronology from this site, which has had its own dendrochronological problems²⁰, seems to have been incorrectly placed. It is now agreed that the German sequence must be broken at 550 BC and the chronology before 550 BC moved back by 71 yr. Once this has been done, consistent cross-dating can be demonstrated between Ireland and Germany at all time periods back to 3000 BC.

A closer look at the critical links within the master chronologies reveals that within the corrected Hollstein chronology there is an overlap of only 33 yr (546–514 BC) and in the south German master chronology an overlap of only 7 yr (546–540 BC). Such overlaps could in themselves never be used as significant links. The weakest link in the Irish sequence is at 250 BC. Here the long floating sequence is linked to the absolute chronology by the Navan-Dorsey series, with overlaps of 130 and 133 yr, respectively. Thus, only by replication between chronologies from the three areas of Ireland, north Germany and south Germany, can final proof of the entire European chronology be provided.

After the German chronology had been broken at 550 BC and the older section moved back by 71 yr, three analyses were carried out to test the replication between the Irish and the corrected German chronologies. The first used the *t*-test²¹ over all the critical overlaps between north Germany and Ireland (Fig. 2). The *t*-test applied here is a rather crude routine test widely accepted as a valuable aid in cross-dating rather than a rigorous statistical test¹⁷. In this program, the correlation coefficient is calculated on the logs of high-pass filtered series and *t* calculated from this. The values of *t* cannot be converted to probabilities because allowance is not made for autocorrelation. The significance of the test in the present study is that in

Fig. 2 Critical links between chronologies from Ireland, England, north and south Germany over the period AD 500 to 1200 BC showing the weak link at 550 BC in the German sequence and at 250 BC in the Irish sequence. The numbers shown beside the links are *t*-values calculated according to Baillie and Pilcher²¹. The unpublished Southwark chronology was provided by Dr I. Tyers. The West German chronology is from Hollstein¹⁹.



all but one of the cases illustrated in Fig. 2 (the exception being Navan-Dorsey and Southwark), the t -value quoted is the highest value for all possible positions of overlap of the two series. The t -test should be seen as complementing the visual cross-dating.

In addition to the t -test, the percentage of parallel variation, GL% (Gleichlaufigkeits value²²), provides a powerful test of synchrony when used on long time series. This simple non-parametric test derives the first differences of the two series and then determines what percentage of the total common years of the two series has the same sign. A test of the south German chronology against the Irish chronology over a total of 4,700 yr gave a GL% of 54.0%, significant at the 99.99% level. If the 1,582 signature years in the south German chronology are tested against the same years in the Irish sequence, the GL% rises to 56.1%, again significant at the 99.99% level. The Irish sequence and the three north German sequences give GL% values of 57.0, 56.8 and 55.6%, all significant at the 99.99% level.

A third confirmatory test was carried out between the Irish and the north and south German chronologies. The long chronologies were split into 400-yr sub-periods, each staggered by 100 yr, and tested using the GL% test. Consistent values (all significant at least at the 95% level, many at 99.99%) were obtained over the entire period from 2000 BC to AD 500. As these correlations are maintained across the weak links at 550 BC and 250 BC, it is possible to claim that a fully replicated European chronology complex exists back to the third millennium BC. The Irish chronology is continuous and internally replicated back to 5289 BC.

Having established on tree-ring grounds that the German chronologies before 550 BC must be moved back by 71 yr, the observed discrepancy between the radiocarbon measurements on bristlecone pine and German oak, found to be between 69 and 72 yr²³, can now be explained. The construction of over 7,000 yr of European tree-ring chronology means that all of the radiocarbon calibration measurements on European oak are now anchored to a calendrical time scale and can be compared directly with measurements on bristlecone pine. The high precision results reported by the Belfast laboratory in 1983 used this time scale^{25,26}. The other implications of the completion of such long sequences of tree rings have yet to be assessed. An increasing number of archaeological sites, particularly in central Europe, can now be given definitive calendrical dates¹²⁻¹⁴. Extension of the European chronology further into the early post-glacial is currently under investigation, and there is a possibility of further replication using another German oak chronology established at the Göttingen laboratory²⁴. The long tree-ring sequences clearly have potential for long-term palaeoclimatic studies not only by palaeoecological²⁷ and climatological^{28,29} interpretation of their growth patterns, but also by their use for radiocarbon³⁰ and stable isotope studies³¹.

We thank the Science and Engineering Research Council for support of the British Isles tree-ring work.

Received 14 May; accepted 5 September 1984.

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Identification of rat γ atrial natriuretic polypeptide and characterization of the cDNA encoding its precursor

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Diuretic and smooth muscle-relaxing peptides, designated atrial natriuretic peptides (ANPs), have been identified in human¹⁻³ and rat⁴⁻¹⁰ atrial tissues and implicated in the control of fluid volume and vascular function^{11,12}. Recently, cDNAs encoding the human² and rat¹³⁻¹⁵ ANP precursors have been sequenced. We previously isolated from human tissue a natriuretic peptide of molecular weight (MW) 13,000 (γ -hANP) comprising 126 amino acid residues, the largest natriuretic peptide so far identified, and showed that it is directly derived from the 151-residue human ANP precursor by the removal of a signal peptide^{2,3}. We now report the isolation and sequence analysis of a novel rat atrial natriuretic peptide (γ -rANP) of MW 13,000, which derives from the rat ANP precursor. We also report the molecular cloning and nucleotide sequence analysis of the cDNA of the 152-residue rat ANP precursor, which is remarkably similar to the human 151-residue precursor (pre-hANP) except at the C-terminus. Differences in the rat and human precursor nucleotide sequences around the termination codons lead to a difference in processing pattern.

γ -rANP was purified from a side fraction isolated previously^{6,9} using an assay for relaxant activity in chick rectum¹². Early in the purification procedure, the γ -component of MW 13,000 was separated from other lower-molecular weight components on the basis of its weaker basicity by batchwise chromatography on SP-Sephadex C-25 of rat atrial extracts^{6,9}. Gel filtration of the γ -component on Sephadex G-75 yielded crude γ -rANP as a major peak of rectum-relaxing activity eluting in fractions 41-47, corresponding to a molecular weight of ~13,000 (Fig. 1a). Subsequent reverse-phase HPLC yielded purified γ -rANP, a 126-amino acid peptide with similar but less potent natriuretic activity than lower-molecular weight α -rANP⁹ and β -rANP⁶. The yield from 258 rat atria (52.5 g), estimated from amino acid analysis, was 115 nmol which is surprisingly higher than those of smaller rANPs^{6,9} (~1-2 nmol each in the present purification), indicating that γ -rANP is the major natriuretic peptide in rat atria.

Sequence analyses of the disulphide-linked γ -rANP were performed after converting to the reduced and S-carboxymethylated (RCM-) derivative. Direct Edman degradation of the purified RCM- γ -rANP identified the N-terminal sequence (1-41, Fig. 2). The C-terminal sequence of the RCM- γ -rANP, verified by carboxypeptidase digestion^{1,3,6}, was Ser-Phe-Arg-Tyr, which is identical to those of the rANPs so far identified,

Fig. 1 *a*, Gel filtration of fraction SP-II, derived from rat atrial extracts on a calibrated column of Sephadex G-75 (1.8×135 cm; flow rate 10 ml h⁻¹; eluent, 1 M AcOH; fraction size, 5 ml per tube). Relative relaxant activity in chick rectum is indicated by black bars. *b*, Reverse-phase HPLC of purified γ -rANP (1.25 nmol). Column: TSK LS-410 ODS SIL (4×250 mm; ToyoSoda). Solvent system: linear gradient elution from (A) to (B) for 80 min; flow rate 1 ml min⁻¹; H₂O/CH₃CN/10% trifluoroacetic acid (TFA) = 90:10:1 (A) and 40:60:1 (B) (v/v). *c*, Reverse-phase HPLC of tryptic digests derived from RCM- γ -rANP (2.5 nmol). For trypsinization conditions see Fig. 2 legend. Column: Chemcosorb 3 ODS-H (4.6×75 mm; Chemco). Solvent system: linear gradient elution from (A) to (B) for 60 min; flow rate 1 ml min⁻¹; H₂O/CH₃CN/10% TFA = 100:0:1 (A) and 40:60:1 (B) (v/v). Tryptic peptides, designated as T, are numbered sequentially in the order in which they occur in γ -rANP (see Fig. 2). T1' is identical to T1, except that Met residue is oxidized.

Methods: The starting material used was a side fraction, referred to as the γ -component of rectum activity corresponding to MW ~13,000, which was obtained in our previous purification of α - and β -rANP^{6,9}. Rat atrial tissue (52.5 g) was extracted from 258 rats and treated as described previously^{6,9} to collect the fraction corresponding to MW <15,000. After lyophilization, dry material was dissolved in 1 M AcOH, then subjected to batchwise chromatography on SP-Sephadex C-25 (H⁺-form), pre-equilibrated with 1 M AcOH. Successive elutions with 1 M AcOH, 2 M pyridine and 2 M pyridine-AcOH (pH 5.0) yielded three fractions: SP-I, SP-II and SP-III. The γ -component was eluted in fraction SP-II, while the α - and β -components eluted in fraction SP-III. SP-II obtained in this manner was the starting material for purification of γ -rANP. Gel filtration of SP-II was performed on a column of Sephadex G-75. An aliquot from each fraction was bioassayed for relaxant activity in chick rectum (*a*). Fractions 41–47 eliciting the rectum activity were subjected to preparative HPLC on a reverse-phase column of TSK LS-410 ODS SIL (8.0×250 mm; ToyoSoda) and rechromatographed. The purity of γ -rANP obtained in this manner was checked by further reverse-phase HPLC (*b*). Bioassay was performed as follows. Chick rectum relaxant activity of the sample was assayed at 37 °C as described elsewhere^{1,12}, using strips of freshly isolated chick rectum. Muscle tone was induced in the rectum strips by adding 2×10^{-8} M carbamyl choline. Natriuretic and diuretic activities after injection into rats were assayed elsewhere^{1,11}. Three observations were made for each dose.

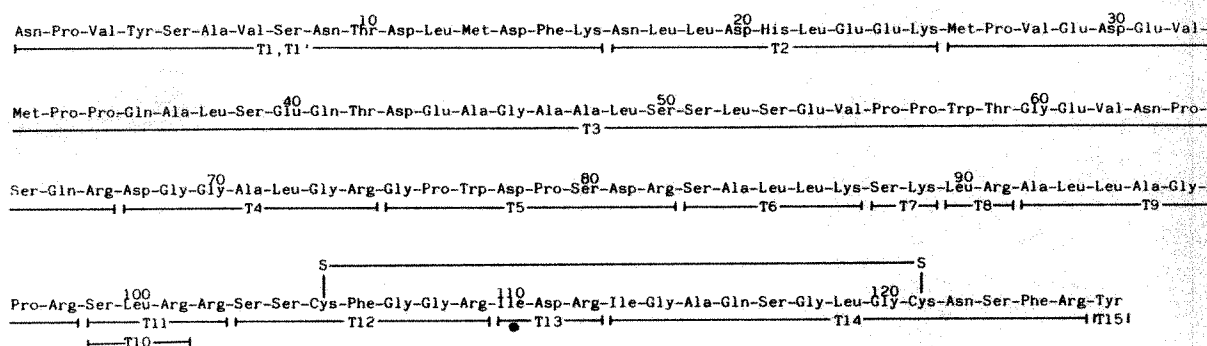
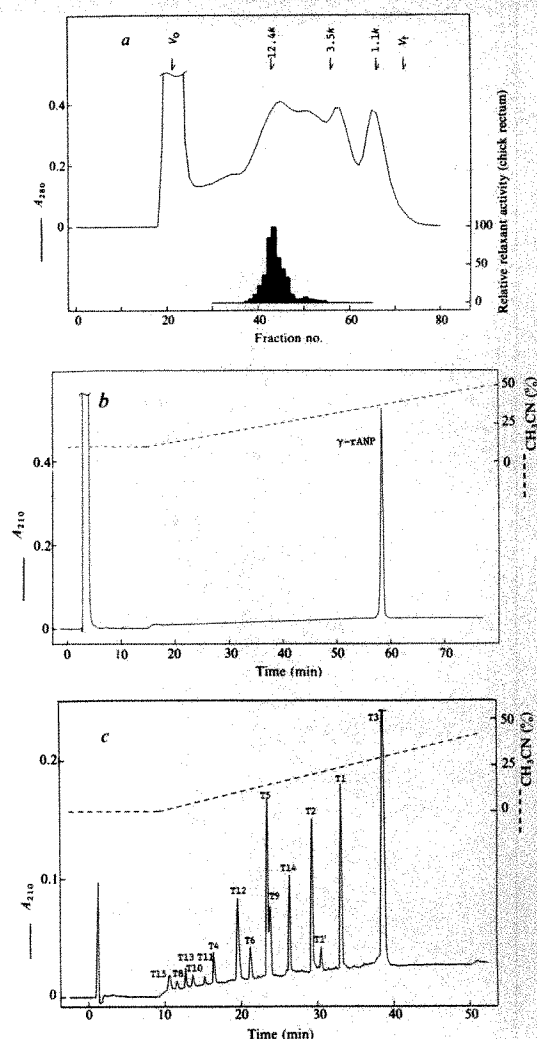


Fig. 2 Complete amino acid sequence of γ -rANP. Sequence analyses were performed with RCM- γ -rANP and a series of tryptic peptide fragments (designated as T). S-carboxymethylation of γ -rANP was achieved by reduction with 50 mM dithiothreitol (DTT) in 0.5 M Tris-HCl buffer (pH 8.5) for 4 h at 37 °C, followed by reaction with 100 mM iodoacetate for 5 min. The resulting RCM- γ -rANP was purified by reverse-phase HPLC. Carboxy-terminal analysis of RCM- γ -rANP (1.0 nmol) was performed by successive digestion with carboxypeptidase A and B (100 ng each; Sigma) in a buffer of 0.2 M N-ethylmorpholine acetate (pH 8.0) at 37 °C. Amino acids released at appropriate time intervals were analysed at the picomole level with a pre-fluorescence labelling analysis system (Waters)^{1,6,9}. RCM- γ -rANP (2 nmol) was digested with 2 μ g of trypsin (tosylphenylethylchloromethyl ketone-treated; Worthington) in 25 μ l of 1% ammonium bicarbonate (pH 8.0) at 37 °C for 3 h. Tryptic peptides were separated by reverse-phase HPLC (Fig. 1c). Sequence analyses of RCM- γ -rANP and its tryptic peptides were performed by stepwise Edman degradation, using a gas-phase automated sequencer (Applied Biosystems 470A), coupled with HPLC identification of the resulting PTH-amino acids.

implying that γ -rANP represents an N-terminally elongated form of the known rANPs.

Further structural information was provided by analysing tryptic peptides derived from RCM- γ -rANP, which were separated by reverse-phase HPLC (Fig. 1c). Overlapping of the sequences of tryptic peptides with the N-terminal (1–41) sequence determined above and the known sequences of 73- (ref. 7) and 48-residue⁶ rANPs made it possible to establish

unambiguously the complete amino acid sequence of γ -rANP (Fig. 2).

We have also cloned and sequenced cDNAs encoding γ -rANP, as described in Fig. 3 legend. Briefly, the hANP cDNA insert which we had previously isolated and analysed was ³²P-labelled by nick-translation and used as a probe for screening a cDNA library constructed from atrial poly(A)⁺ RNA of Wistar Kyoto rats. The nucleotide sequence of plasmid prANP10 which

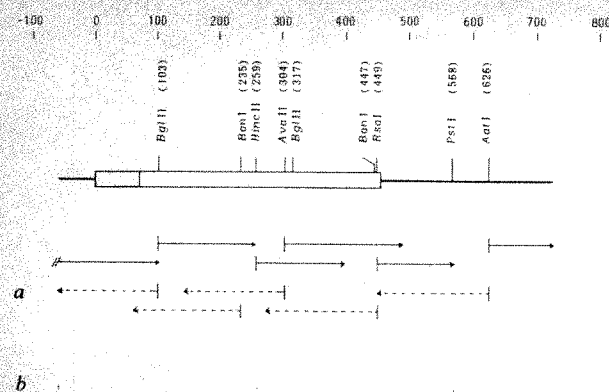


Fig. 3 Sequencing strategy for the cDNA insert of prANP10. The restriction map shows only those restriction sites relevant to the present work; they are identified by numbers indicating the 5'-terminal nucleotides generated by cleavage. □, Sequence corresponding to coding region; ■, coding region for putative signal peptide. Nucleotides are numbered as in Fig. 4. The direction and extent of sequence determination are shown by arrows (→, upper strand; ←, lower strand). The sites of 5'-end-labelling are indicated by the short vertical lines at the ends of arrows. Slash marks mean that 5'-end-labelling was done on the site located on the vector DNA. Vertical dotted lines mean that the transcription primed by the general primers was started from the sites located on the pUC8 plasmid.

Methods: A cDNA library was constructed by the method of Okayama and Berg²² from 4.2 µg of the vector-primer and 20 µg of poly(A)⁺ RNA isolated from 100 atria of Wistar Kyoto rats as before. Approximately 10,000 transformants of *Escherichia coli* WA802 were obtained from ~1.7 µg of the original poly(A)⁺ RNA; these were screened by hybridization²³ with ³²P-labelled probe at 65 °C for 16 h. The probe was prepared from a 463-bp *EcoRI*-*PstI* fragment of the cDNA insert of phANP82 which had been cloned from a library of cDNA of human atrial total poly(A)⁺ RNA as described previously³. Positive clones were submitted to cleared lysate analysis^{3,24}, and we confirmed that the three clones (prANP10, 11 and 12) which had a long cDNA insert contained the α-rANP coding sequence by hybridizing with tetradecamer pools (oligo I and II), representing all possible complementary sequences corresponding to α-hANP(12-16), which were used to probe cloned cDNA encoding the human precursor³. The plasmid prANP10 was used for further sequencing as described previously³: a, Maxam-Gilbert method²⁵; b, method of Sanger *et al.*²⁴, after cloning three fragments of the insert (*PstI*-*HincII*-*PstI* and *PstI*-*PvuII*, generated by digestion with *PstI* and *PvuII* endonucleases) into plasmid pUC8. (The Okayama-Berg primer-vector carries *PstI* and *PvuII* sites.)

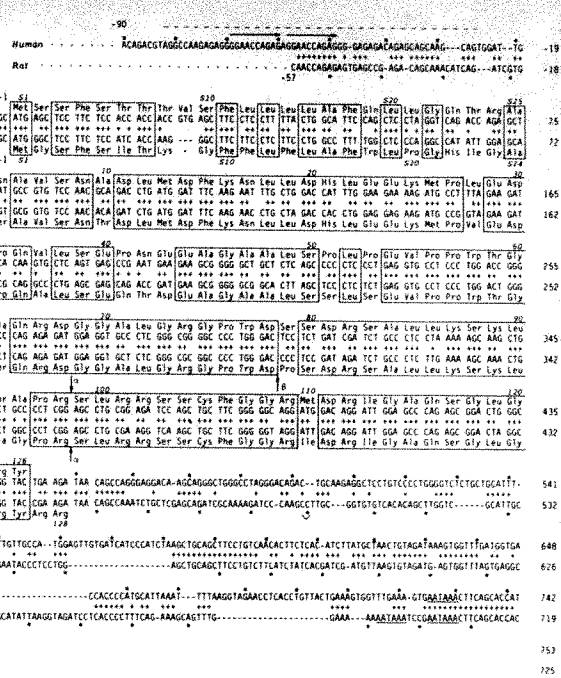


Fig. 4 Nucleotide and deduced amino acid sequences of the cDNA for pre-rANP, compared with those of pre-hANP cDNA³. The nucleotide sequence of prANP10 was determined as described in Fig. 3 legend; the predicted amino acid sequence is shown under the nucleotide sequence. The nucleotide and amino acid sequences of the pre-hANP cDNA³ are shown above those of the pre-rANP cDNA for comparison. Nucleotide residues are numbered (Roman type) in the 5' to 3' direction, beginning with the first residue of the ATG triplet encoding the initiating methionine. Nucleotides 5' of residue 1 are indicated by negative numbers. Amino acids are numbered in italics starting from the initiating methionine and the N-terminal residues of γ-ANPs; S before the number denotes the putative signal sequence. Sequences are aligned to give maximum homology with the introduction of minimal deletions. Deletions are indicated by short horizontal lines on a one-to-one basis. Identical amino acids and the triplets encoding them are boxed; identical nucleotides are marked by +. Asterisks above or below nucleotides of untranslated regions are marker nucleotides. The broken line at the top indicates a sequence which has no thymidylate residues; horizontal arrows indicate direct repeats. Vertical arrows labelled with Greek letters indicate cutting sites used to generate the respective ANP subclasses. AATAAA sequences are underlined. Poly(A) stretches and their approximate chain lengths are shown by (A) and suffixes.

had the longest insert (782 base pairs (bp), excluding the poly(A) sequence), was found to contain the entire coding sequence for γ-rANP (Fig. 4). The N-terminal 24 amino acids of the rat ANP precursor (pre-rANP), share characteristic features with the signal sequences generally found in presecretory proteins^{16,17}. It has many hydrophobic amino acids, indeed residues S10-S19 are all hydrophobic, forming a hydrophobic core¹⁸. The apparent cleavage site for signal peptidase is after residue 24, alanine, which often occurs before cleavage sites for other signal peptidases¹⁸. The sequence encoding γ-rANP of 126 amino acid residues, immediately follows the putative signal peptide sequence and ends at residue 150, a tyrosine (amino acid 126), which is connected to the extra C-terminal Arg-Arg sequence. Note that in pre-hANP, translational termination occurs just after the last residue, a tyrosine. Interestingly, 15 nucleotides downstream, the codon for the C-terminal tyrosine of pre-hANP (position 126) is identical to that of pre-rANP, except for a single base replacement at the first nucleotide, which converts the termination codon TGA in pre-hANP to the codon for Arg in pre-rANP. Thus, translation of pre-rANP continues two amino acid residues beyond Tyr 126 and ends at the other termination triplet, TAA. The absence from rat atrial extracts

of the peptides that terminate in the Arg-Arg sequence suggests that the C-terminal Arg-Arg is removed from the 152-residue precursor at an earlier stage after synthesis to produce γ-rANP of 126 amino acids. The difference is not due to an error during reverse transcription because the sequence of another plasmid (prANP12), which is shorter than that of prANP10, was found to have the same sequence.

The human ANP precursor contains no glycosylation sites¹⁹. In contrast, the rat precursor contains the tripeptide sequence Asn-Pro-Ser, which is regarded as a possible glycosylation site, at positions 63-65 (Fig. 2). However, no glycosylation at Asn 63 was detected in γ-rANP.

There is remarkable homology between the human and rat γ-ANPs in both the nucleotide (85.2%) and amino acid (86.5%) sequences. The amino acid sequences of the putative signal peptides are only 52% homologous, while nucleotide sequence homology is 70.7% if the precursor sequences shown in Fig. 4 are aligned to allow maximum homology. The 5'- and 3'-untranslated regions are very similar. The eight nucleotides upstream of the initiator codons are identical, including the A in position -3 (ref. 20), and the 15 residues downstream from the codons for tyrosine 126 are the same except for the one base as discussed

above. The pre-rANP cDNA sequence has two AATAAA sequences, which precede poly(A) addition sites in many eukaryotic mRNAs²¹, 4 nucleotides apart, whereas the pre-hANP sequence has only one, in the long sequence shared by both precursors. The poly(A) stretch starts immediately after this sequence in the case of pre-rANP, and 5 nucleotides after in the case of pre-hANP. In the 5'-noncoding region of the pre-hANP cDNA insert, GGAACCAGAG sequences are repeated only one nucleotide apart in a long thymidylate-minus sequence. AACCAGAG is also present in pre-rANP cDNA at similar sites, but whether it is repeated or not is unclear because of the apparent incomplete reverse transcription of the mRNA.

Despite the remarkable structural similarities between the

human and rat ANP precursors, their processing is strikingly different. In the human, only three distinct ANPs, α - (28 residues), β - (56 residues) and γ -hANP (126 residues), have been identified¹⁻³. In rat atrial tissue, however, a variety of low- and intermediate-size natriuretic polypeptides (73, 48, 35, 33, 32, 31, 28, 25, 23 and 21 amino acids long) varying in the length of N-terminal extension, has been identified⁴⁻⁹, although in very low yields. The difference in processing patterns may be a result of the amino acid differences between the rat and human γ -ANPs, which may change their conformation and therefore their susceptibility to the proteolytic enzymes, or, more likely, a result of a difference in the enzymatic systems responsible for the processing.

Received 5 June; accepted 20 August 1984.

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Electron microscopic autoradiographic localization of opioid receptors in rat neostriatum

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The regional distribution of opioid receptors in the mammalian central nervous system has been extensively studied by light microscopic autoradiography¹⁻³. Little is known, however, about the fine structural localization of these receptor sites apart from the fact that they are present in both synaptosomal and smooth microsomal subcellular fractions of whole brain homogenates⁴⁻⁶. Previously^{7,8}, using the Met-enkephalin analogue FK 33-824 (Sandoz)⁹, we determined that brain opioid binding sites could be visualized by electron microscopic autoradiography. We now report on the ultrastructural distribution of these sites in the caudate-putamen of the rat. The vast majority of specifically labelled opioid binding sites were found to be associated with axo-dendritic (53%), axo-axonic (18%) or axo-somatic (3%) neuronal membrane interfaces. Only 7%, however, were associated with synaptic junctions. These results suggest that opioids act primarily at non-junctional interfaces on the dendrites, axons and soma of neurones in the neostriatum.

Opioid binding sites in rat brain were labelled *in vitro*, using the monoiodinated opioid pentapeptide agonist FK 33-824 (¹²⁵I-FK)⁸ (see Fig. 1 legend). Film autoradiographs from sections incubated with ¹²⁵I-FK alone revealed a patch-like distribution of bound ligand molecules similar to that previously reported for opioid receptors in rat neostriatum^{10,11} (total binding; Fig. 1a). In contrast, sections incubated in the presence of 1 μ M naloxone showed sparse and homogeneous labelling (non-specific binding; Fig. 1b). Both radioactivity measurements and grain counts performed in light microscopic autoradiographs yielded ratios of specific over total binding of the order of 80%.

In electron microscope autoradiographs from sections incubated with ¹²⁵I-FK alone, most of the silver grains (65.3%) were

found by 50% probability circle analysis^{12,13} to be associated with two or more juxtaposed neuronal elements. Radioactive sources were ascribed by 'line source' analysis¹³ to the apposed plasma membranes (70% of the grains within one half distance of the membranes in a sample of 100 shared grains). However, because of limitations inherent to the autoradiographic technique, it was not possible to determine whether radioactive sources were linked to a particular apposed membrane, or to cell surface rather than to internal membrane components. In sections incubated in the presence of 1 μ M naloxone, silver grains were found predominantly over single neuronal structures and their overall distribution was significantly different from that of total binding. The ultrastructural distribution of speci-

Table 1 Ultrastructural distribution of real and hypothetical grains in autoradiographs from ¹²⁵I-FK-labelled striatal sections

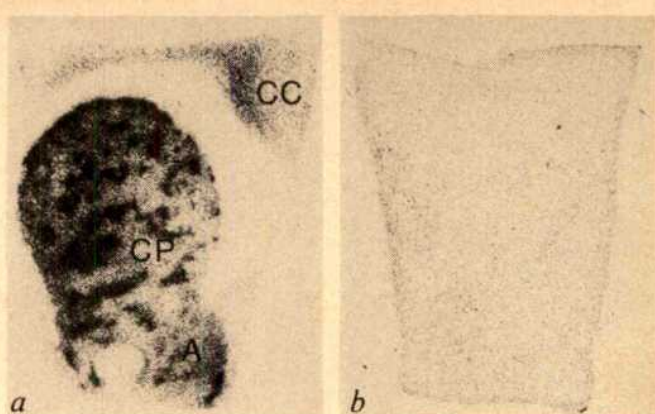
Included within a 50% probability circle	Real (specific) (%)	Hypothetical (%)
Single structures		
Nerve cell bodies	13.1 $\pm$ 1.4	23.3
Axon terminals	1.5 $\pm$ 0.5	12.1
Dendrites	5.9 $\pm$ 3.1	18.7
Apposed structures		
Axon/dendrite:		
Non-junctional	45.8 $\pm$ 3.7	13.0
Junctional	7.1 $\pm$ 0.5	3.1
Axon/axon	17.6 $\pm$ 1.3	9.2
Axon/soma	3.2 $\pm$ 1.1	1.0
Dendrite/dendrite	1.9 $\pm$ 0.5	6.1
Others	3.9 $\pm$ 0.8	13.5
No. of grains counted	1,961	10,039

The distribution of specifically bound ¹²⁵I-FK molecules (real grains) was derived from grain counts performed in sections incubated with ¹²⁵I-FK alone. Values were corrected for nonspecific binding according to the distribution of silver grains in sections incubated in the presence of 1 μ M naloxone. The data are expressed as the mean $\pm$ s.e.m. of five different experiments. Silver grains were ascribed to underlying cellular constituents using a 50% probability circle (diameter = 3.4 $\times$ half distance (HD); HD = 90 nm)¹³. The uniform distribution pattern (hypothetical grains) was generated by means of a transparent overlay displaying a regular array of resolution circles. A χ^2 analysis showed the two distributions to be significantly different with $P \leq 0.005$.

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Fig. 1 Film autoradiographs of 75 μm -thick pre-fixed, vibratome-cut sections from rat neostriatum incubated with 1 nM ^{125}I -FK in the absence (total binding, *a*) or the presence (nonspecific binding, *b*) of 1 μM naloxone, fixed in glutaraldehyde, post-fixed in OsO_4 and dehydrated in ethanol. The distribution of total binding (*a*) conforms with that previously reported for opioid binding sites within the same region^{10,11}. Note the patches of high labelling density in the caudate-putamen (CP) as well as the moderate to strong reactivity of the nucleus accumbens (A) and of the deeper half of layer VI in the cingulate cortex (CC). Nonspecific labelling in the same area (*b*) appears weak and homogeneous by comparison. $\times 7$.

Methods: Adult male Sprague-Dawley rats (150–200 g) were anaesthetized with pentobarbital and perfused for 15–20 min through the ascending aorta with ice-cold 0.1 M phosphate buffer containing 1% tannic acid, 0.75% paraformaldehyde and 0.1% glutaraldehyde. The brains were rapidly removed and serial 70–75 μm -thick sections of the caudoputamen were cut in ice-cold 0.12 M phosphate buffer using a vibrating microtome (Vibratome; Oxford Co.). These sections were incubated with 1 nM monoiodinated ^{125}I -FK 33-824 (100–150 Ci mmol^{-1}) in 0.05 M Tris-HCl containing 0.25 M sucrose pH 7.4, for 30 min at room temperature. The labelled sections were then rinsed twice in cold buffer, fixed with 4% glutaraldehyde, post-fixed in 2% osmium tetroxide and dehydrated in ethanol. This fixation/dehydration sequence was shown to ensure regionally proportional retention of >50% of the bound radioactivity⁸. After dehydration, the sections were placed in a γ counter for determination of the bound radioactivity and either autoradiographed whole using LKB Ultrafilm or flat-embedded in Epon for light and electron microscopic autoradiography. To assess the proportion and distribution of nonspecific binding, some of the sections were incubated with ^{125}I -FK as above, but in the presence of 1 μM naloxone or non-radioactive FK. Specific binding was defined as the difference between total and nonspecific binding. Projection of film autoradiographs over camera lucida drawings of adjacent Epon-embedded sections allowed precise sampling of receptor-dense areas for high-resolution studies. For light microscopic autoradiography, semi-thin (1 μm -thick) sections were taken from the surface of each block and coated by dipping into Kodak NTB-2 nuclear emulsion. For electron microscopic autoradiography, silver-gold sections were cut from the same blocks, collected on parlodion-subbed slides, double-stained with uranyl acetate and lead citrate, sprayed with carbon and coated with Ilford L-4 emulsion diluted 1:4.



cally bound ^{125}I -FK was in turn significantly different from that of uniformly distributed hypothetical grains (Table 1).

Fifty-three per cent of silver grains corresponding to specifically bound ^{125}I -FK molecules were associated with axo-dendritic interfaces (Table 1). Such interfaces were characterized by a close apposition of axonal and dendritic membranes without intervening glial processes (Fig. 2*a, b*). Axonal elements corresponded in most instances to small varicosities (0.75 μm in mean diameter) containing numerous small, round, electron-lucent vesicles (Fig. 2*a, b*) and occasional large granular vesicles. Their dendritic counterparts were mainly shafts of medium to small calibre (Fig. 2*a, b*), but also comprised proximal trunks and spines. In most instances (45.8% of grains counted; Table 1), no synaptic differentiation was present within the resolution circle (Fig. 2*a*). The few labelled contacts exhibiting well defined junctional complexes (7.1% of grains counted; Table 1, Fig. 2*b*) were mostly asymmetrical and were generally established on dendritic spines. Labelled symmetrical junctions were rare

and found solely on dendritic shafts. Several dendritic profiles were labelled at different loci along the plasma membrane (Fig. 2*b*), suggesting that the receptors might be localized on the dendrites themselves rather than on the adjoining axons.

Axo-axonic appositions accounted for the second largest source of specifically labelled ^{125}I -FK binding sites (Table 1). Such appositions included either two axon terminals or one axon terminal and one or more axons. Although a streaming of synaptic vesicles towards the axonal interface was occasionally visible in one of the terminals involved, synaptic differentiations were never observed at the site of contact. One of the terminals, however, was often in synaptic contact with a dendritic profile, generally a dendritic spine (Fig. 2*c*). Previous studies have indicated that selective lesioning of nigrostriatal dopaminergic fibres results in a significant decrease in the number of opioid receptors in rat caudoputamen, suggesting that many of these receptors might be localized on dopamine terminals^{14–18}. The axo-axonic binding sites visualized in the present study may

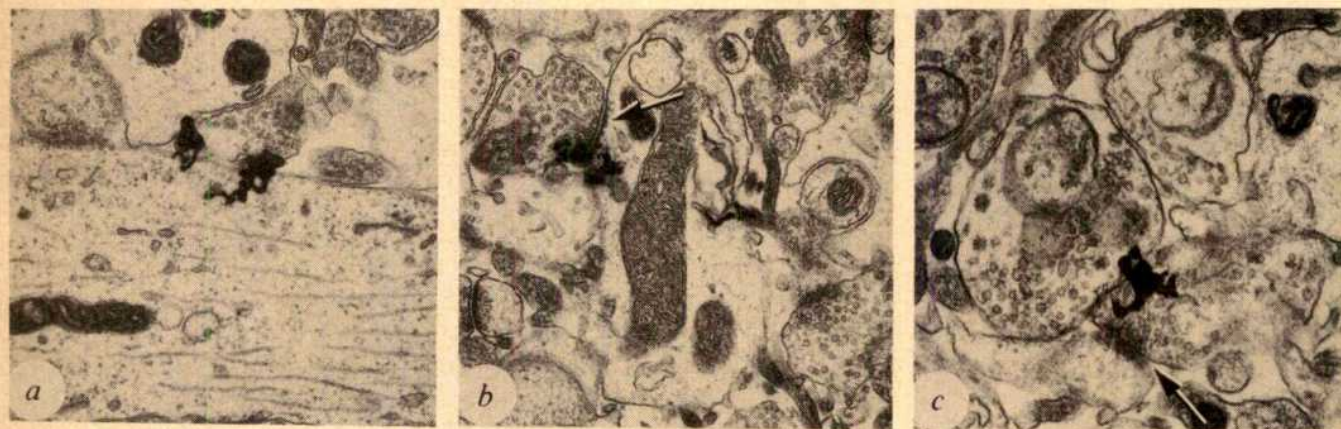


Fig. 2 Electron microscopic autoradiographs from sections incubated with 1 nM ^{125}I -FK alone. The radioactivity is detected in the form of individual silver grains which were ascribed to underlying cellular constituents by means of 50% probability circles (see Table 1). In all cases illustrated here, the 50% probability circle encompasses two neuronal structures and the apposition of their respective plasma membranes. *a*, These two silver grains are associated with a non-junctional axo-dendritic interface involving a small axon terminal filled with numerous small clear synaptic vesicles. $\times 21,000$. *b*, The two silver grains on the left are associated with an asymmetrical axo-dendritic synaptic junction. A third silver grain on the right overlies the plasma membrane of the receiving dendrite. $\times 19,000$. *c*, This silver grain is localized at the interface of two axon terminals. Note the absence of synaptic specialization at the site of apposition. The synapse established on a dendritic spine by one of the terminals involved was not included within the resolution circle. $\times 26,000$. Development, D-19; exposure time, 10 weeks.

correspond to some of these postulated axonal receptors and could thus mediate, at least in part, the demonstrated effects of opioids on the striatal release of dopamine^{19,20}.

Finally, some of the labelled binding sites were associated with axo-somatic appositions (Table 1). Occasionally, a symmetrical synaptic differentiation was present at the site of contact between the axon terminal and the cytoplasmic membrane of the cell. Most of the nerve cells involved were of medium size (12–22 μm in diameter), with a large nucleus showing very little or no indentations and a small rim of surrounding cytoplasm containing very few organelles. These neurones were classified as spiny type 1 neurones, based on cytoarchitectonic analyses of rat neostriatum^{21,22}.

Taken together, the present results strongly suggest that ¹²⁵I-FK-labelled opioid receptors are primarily involved in the mediation or modulation of axo-dendritic, axo-axonic and, to a lesser extent, axo-somatic neural transmission in the rat neostriatum. The identity and site of release of the endogenous ligand(s) acting on these receptors remain to be determined. ¹²⁵I-FK labels both μ and δ opioid receptors^{23–25}, for which enkephalins have been proposed as natural endogenous ligands. Enkephalins could be released by terminals that either possess or are immediately adjacent to ¹²⁵I-FK binding sites, as suggested by the similarity, with respect to size, cytological features and synaptic relationships, between the terminals labelled in the present study and enkephalin-containing terminals previously identified in rat neostriatum by immunocytochemistry^{26,27}. Enkephalins could also be released at a distance from the labelled binding sites and diffuse in tissue to act on a relatively widespread population of receptors; this would account for the apparent lack of correlation between the distribution of enkephalin terminals and opioid receptors in rat striatum (see ref. 28). In any event, endogenous as well as exogenous opioids are likely to act primarily on non-differentiated membrane targets in rat caudate-putamen. Indeed, a major finding of the present study is that only a small proportion of the labelled binding sites was actually associated with synaptic junctions. This is in contrast to α -bungarotoxin^{29–31}, muscarinic³² and benzodiazepine³³ binding sites visualized by electron microscopy or horseradish peroxidase conjugation in rat central nervous system, for all of these have been found to be predominantly, although by no means exclusively, associated with synaptic contacts. That interneuronal communication can occur in the absence of membrane specialization has already been postulated for monoaminergic^{34,35} and peptidergic^{36–38} neurotransmission in the central and peripheral nervous systems. Correlative biochemical and immunohistochemical data³⁹ have indicated that opioids, in particular, may act non-synaptically in rat neostriatum, dorsal horn of the spinal cord and spinal trigeminal nerve. The non-junctional receptors visualized in the present study provide the first direct evidence for such a mode of action.

We thank Kathleen Leonard and Charles Hodge for technical assistance, Dr Michael Dennis for his help with the iodination of the peptide, Dr Barbara E. Jones for reviewing the manuscript, and Beverley Lindsay for typing it. The drug FK 33-824 was kindly provided by Dr Dietmar Roemer (Sandoz). This work was supported by a fellowship (E.H.), a scholarship (A.B.) and grant MA-7366 from the MRC of Canada.

Received 6 March; accepted 10 September 1984.

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Hybrid HLA-DC antigens provide molecular evidence for gene *trans*-complementation

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In the mouse there are hybrid determinants for the immune region (*Ir*) of the genome which contribute to the diversity of class II (*Ia*) antigens of the major histocompatibility complex (MHC) and provide a molecular basis for *Ir* gene complementation^{1–4}. In man, two prominent families of Ia molecule, HLA-DR and HLA-DC (or DS)^{5,6}, have been identified which are respectively homologous to the murine I-E (E_α , E_β) and I-A (A_α , A_β) antigens⁷. Whereas DR antigens consist of a constant α -chain and an extremely polymorphic β -chain⁸, both the α and β subunits of DC antigens are structurally variable when different alleles are compared^{9,10}. The marked differences in the structure of the α - and β -chains of HLA-DC molecules result in electrophoretic variants which allow the haplotype of origin of each subunit to be identified by two-dimensional gel electrophoresis. We report here that gene *trans*-complementation occurs in cells heterozygous for the HLA-D region, resulting in the expression of hybrid HLA-DC molecules in addition to the parental forms. Generation of new HLA-D region hybrid molecules contributes to the qualitative diversity of human MHC class II antigens and has important functional implications in immune regulation.

HLA-DC molecules radiolabelled with ³⁵S-methionine were isolated by indirect immunoprecipitation from detergent-solubilized membranes of three cell lines—SC-CA (DR1 homozygous), GM 3163 (DR7 homozygous) and LES (DR1/DR7 heterozygous Epstein-Barr virus-transformed)—and a set of monoclonal antibodies previously reported to recognize HLA-DR and/or HLA-DC determinants was used to isolate and characterize the different HLA-DR and HLA-DC molecules present in the cell lines. Monoclonal antibody Genox 3.53 (ref. 11) recognizes the DC1 antigen¹² expressed on DR1-2- or W6-positive cells. As reported previously, the N-terminal amino acid sequence of the DC1 molecule isolated using this antibody is homologous to those of the murine I-A antigens for both the α - and β -chains^{5–13}. Immunoprecipitation of SC-CA

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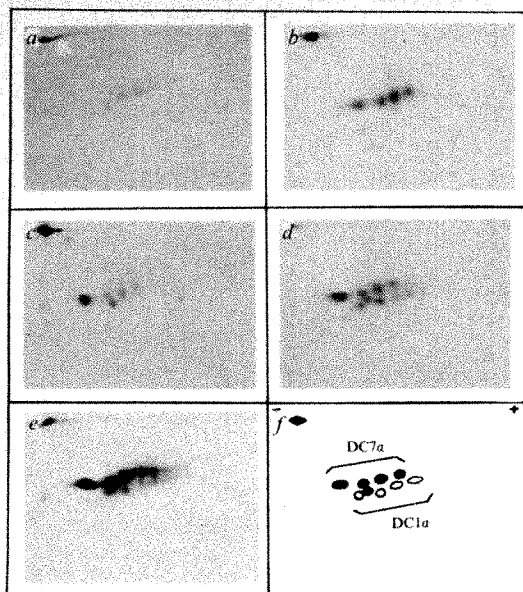


Fig. 1 Comparative IEF two-dimensional PAGE profiles of the DC α -chains from SC-CA (*DR1/DR1*), GM 3163 (*DR7/DR7*) and LES (*DR1/DR7*) cell lines. *a*, The DC1 α -chain isolated from SC-CA using the Genox 3.53 (anti-DC1) monoclonal antibody. 'A' indicates the position of actin (43,000 molecular weight). *b*, The DC1 α -chain from LES immunoprecipitated with Genox 3.53. *c*, The DC7 α -chain from GM 3163 DR⁻ immunoprecipitated with SG 171 or SG 520 is slightly higher and more basic. *d* Illustrates the ability of SG 171 to immunoprecipitate DC7 plus DC1 α -chains in LES DR⁻. The above pattern is identical to that obtained by mixing the DC1 α -chain from SC-CA and the DC7 α -chain from GM 3163 (panel *e*). *f*, Schematic representation of the DC1 α (○) and DC7 α (●) chains IEF20 PAGE patterns.

Methods: Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum and labelled with ³⁵S-methionine in methionine-free medium as described in detail elsewhere¹⁵. The membranes were extracted with 0.5% Nonidet P-40 (NP40). The immunoprecipitated samples were separated by IEF two-dimensional PAGE. The ampholine mixture used in IEF consisted of pH 3.5 ampholine (0.5%) and pH 5-7 ampholine (1.5%). The second dimension was a 10% polyacrylamide slab gel. The panels show portions of autoradiographs of IEF two-dimensional PAGE of the immunoprecipitated proteins. The DC α -chains consist of four or five acidic spots.

(*DR1/DR1*) with Genox 3.53 enabled us to isolate the DC1 molecule; Genox 3.53 does not react with GM 3163. Two other monoclonal antibodies, SG 171 and SG 520, were used to characterize DC7 antigens. SG 171 was originally found to recognize I-A-like molecules from marmoset cell lines¹⁴. However, two-dimensional polyacrylamide gel electrophoresis (PAGE) and partial amino acid sequence data indicate that SG 171 displays an unusual pattern of reactivity for human Ia antigens. In addition to reacting with HLA-DR antigens of all the different alleles, including the *DR7* allele, SG 171 cross-reacts with the HLA-DC molecule present in *DR7* homozygous cells⁶. SG 171 is thus suitable for isolating the DC7 molecule after complete removal of the HLA-DR antigen. This was achieved through several cycles of immunoprecipitation with the monomorphic anti-HLA-DR monoclonal antibody, 1.41 (ref. 15). Antibody SG 520 has recently been reported to react only with DC7 molecules when used on cell lines that are *HLA-DR7* homozygous¹³. In the present work, the HLA-DC7 molecules were isolated by immunoprecipitation with SG 520 or SG 171 after depletion of HLA-DR. (We will refer to cell lysates from GM 3163 (*DR7/DR7*) and LES (*DR1/DR7*) depleted of HLA-DR antigen as GM 3163 DR⁻ and LES DR⁻, respectively.)

The immunoprecipitated HLA-DC antigen was analysed by two-dimensional PAGE as described previously. The use of non-equilibrium pH gradient electrophoresis in the first dimension provides a good separation of the β -chains of both

the HLA-DR⁸⁻¹⁵ and HLA-DC molecules⁶⁻¹⁴. However, the α -chains were poorly resolved by this procedure⁶⁻¹⁴, and we therefore used electrophoretic conditions known to maximize resolution of the acidic area of the gel. Analysis of the DC α -chain by isoelectrofocusing (IEF) two-dimensional PAGE in place of non-equilibrium pH gradient two-dimensional-PAGE revealed an acidic series of four or five discrete spots. This pattern is reproducible for a given DC α -chain. Furthermore, the α -chain species could be precisely located according to their position relative to the position of actin, a 43,000-molecular weight protein present in all MHC class II immunoprecipitates²⁻¹⁵. The DC1 α -chains isolated from SC-CA and LES cell lines using Genox 3.53 were indistinguishable by their IEF two-dimensional-PAGE profile (Fig. 1*a,b*). This complete identity was confirmed when a mixture of the two immunoprecipitates was analysed simultaneously on the same gel. Compared with the DC1 α -chain, the α -chain of the DC7 antigen isolated from GM 3163 as described above is slightly higher and more basic (Fig. 1*c*). The DC7 α -chain IEF two-dimensional-PAGE profiles were identical when GM 3163 was immunoprecipitated with antibody SG 520 or when another *DR7* homozygous cell line (Mann) was used. Structural polymorphism among *DC* α alleles has been reported previously using amino acid sequence data, DNA restriction fragment analysis and peptide mapping^{6,10,16}. Based on these results, we searched for electrophoretic variants of the DC α -chains. Because the IEF two-dimensional-PAGE patterns of DC α -chains are reproducible and distinguish between alleles, they can be used as molecular fingerprints to identify the haplotype of origin of the spots and therefore provide a method for typing the *DC* α locus.

Because IEF two-dimensional-PAGE analysis demonstrated haplotype specificity for the DC α -chains we used antibody SG 171 to investigate the DC antigens present in a cell lysate (LES DR⁻) from a *DR1/DR7* heterozygous individual carrying *DC7* on one chromosome and *DC1* on the other chromosome; this antibody would be expected to react only with the *DC7* species of DC molecule. However, the PAGE pattern of SG 171-reactive molecules isolated from LES DR⁻ cells revealed a more complex pattern than expected for the *DC7* α -chain (Fig. 1*d*); it represented the combined pattern of the DC1 and *DC7* α -chains. In fact, the pattern was identical to that obtained when DC1 and *DC7* antigen preparation were independently isolated from a *DR1* and a *DR7* homozygous cell respectively and co-electrophoresed on the same gel (Fig. 1*e*). We conclude from this that a monoclonal antibody possessing a biochemical specificity restricted to the *DC7* antigen is able to precipitate a DC1 α -chain associated with the *DC7* molecule in a *DR1/DR7* heterozygous cell line.

Several hypotheses were investigated to explain the presence of both α -chains. The DC1 α -chain could not carry a determinant recognized by SG 171 because the antibody would then be expected to immunoprecipitate DC1 from SC-CA (*DR1/DR1*), which it does not. This suggests that the DC1 α -chain is precipitated by SG 171 through its association with components of the *DC7* antigen. Because non-equilibrium pH two-dimensional PAGE reveals only one β -chain, corresponding to the *DC7* β -chain, our observation suggests that the *DC7* β -chain freely associates with both the *DC7* and DC1 α -chains. Furthermore, depletion of the *DR1/DR7* heterozygous cell lysate with antibody Genox 3.53 removes the complete DC1 molecule (DC1 α , DC1 β) but does not result in a decrease of the DC1 α -chain precipitated with SG 171, thus confirming the presence of an additional molecular species that is independent of the DC1 complex. Because the Ia antigen consists of two closely associated chains, an antibody to either chain would precipitate the α - β dimer. To investigate the possibility that the antibody reacted only with the *DC7* β -chain, isolated HLA-DC7 antigens from GM 3163 were precipitated, separated by SDS-PAGE in non-dissociating (unboiled) and dissociating conditions, electrophoretically transferred to nitrocellulose membranes and incubated with dilutions of antibody SG 171 and ¹²⁵I-protein A (Fig. 2). We found that antibody SG 171 bound strongly to the

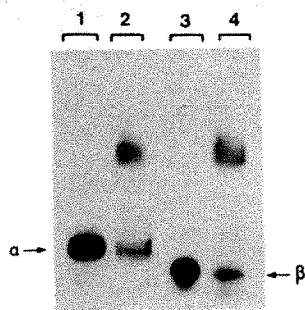


Fig. 2 Detection of DC α -chain specificity of monoclonal antibody SG 171 by electrophoretic transfer of proteins from SDS-PAGE to nitrocellulose. Soluble membrane proteins from unlabelled GM 3163 cells were extracted at 10^8 cells ml^{-1} in 0.5% NP40 Tris-buffered saline (150 mM NaCl, 50 mM Tris, 0.02% NaN_3 , pH 7.0) for 15 min at 4°C. The insoluble material was removed by centrifugation at 100,000g for 60 min. The HLA-DR antigens were retained through eight cycles of immunoprecipitation with antibody 1.41, a monomorphic anti-HLA-DR and *Staphylococcus aureus* A protein¹⁵. The cell proteins depleted of DR reactivity were separated on a 10% polyacrylamide slab gel in non-dissociating conditions (no boiling) and dissociating conditions. Unlabelled polypeptides were transferred to a nitrocellulose membrane. After transfer, the filter was incubated with a 1:1,000 dilution of SG 171 and ^{125}I -protein A. DA 147 (ref. 17), an antibody directed against the α -chains (DR and DC), was used as control. DA 147 reacts with the complex (lane 2) and after dissociation only with the α -chain (lane 1). In contrast, SG 171 reacts with the complex (lane 4) and only with the DC β -chain (lane 3). Reactivity with the separated chains (α and β) in lanes 2 and 4 is the result of a low level of chain dissociation which occurs even without boiling the sample.

dimer and to only the β -chain when the complex was dissociated. An antibody directed against the α -chain (DA 147) was used as a control¹⁷.

From the above results, we conclude that in a heterozygous cell line, a DC β -chain can associate freely with the DC α -chain of either maternal or paternal origin, thus generating mixed-haplotype hybrid molecules. Four possible combinations could be generated by the different associations between the α - and β -chains of the DC loci and would therefore be expressed by individual cells. Several laboratories have presented serological evidence that in the mouse, certain Ia determinants exist as hybrid Ia molecules which are expressed on lymphocytes from F_1 animals but are not expressed on cells of either parental strain^{18,19}. Furthermore, combinatorial antigens can function effectively as in the mixed lymphocyte reaction and as restriction elements for T-cell recognition of antigen-specific clones^{1,20,21}. Jones *et al.*² and Silver²² have shown in biochemical studies that one gene product of the I-A region (A_β) and a gene product of the I-E region (E_α) could interact in either the *cis* or *trans* configuration in an F_1 animal. Similar associations occur between the A_α and A_β polypeptides in the appropriate F_1 , as demonstrated by Silver *et al.*³. No convincing serological or biochemical data for an HLA-D region hybrid antigen in humans are available. However, Hansen *et al.*²² observed HLA-D region *trans*-complementation in human T-cell clones that responded to tuberculin only when presented by accessory cells carrying both HLA-D region antigens possessed by the autologous cell donor²². Our finding that DC α - and DC β -chains from a heterozygous cell are capable of associating to form a novel molecule that may carry unique determinants present in the heterozygous cell and absent from both the maternal and parental haplotype may provide a molecular explanation for this. The latter data suggest the presence of new cell-surface molecules functioning as restriction elements independently of whether they originate from the maternal or paternal chromosome. Because the DR α -chains from different alleles are non-polymorphic, the haplotype of origin of the DR α -chain associ-

ated with a given DR β -chain should not be responsible for generating new Ia determinants. In contrast, the variability of the DC α -chain probably creates determinants of mixed haplotype. Such T-cell clones could represent the functional counterpart of the hybrid DC molecules described here.

Our results describe an additional source of structural diversity of the human Ia antigens which are expressed in HLA-D region heterozygous individuals and thus indicate that the number of potential Ia restriction sites is much greater than the number of Ia molecules encoded in the genome.

We thank F. Brodsky and P. Parham for the gift of Genox 3.53, J. Silver and S. Goyert for SG 171 and SG 520 and V. Van Heyningen and M. Steel for DA 147.

Received 20 February; accepted 22 August 1984.

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Tissue-specific transcription preference as a determinant of cell tropism and leukaemogenic potential of murine retroviruses

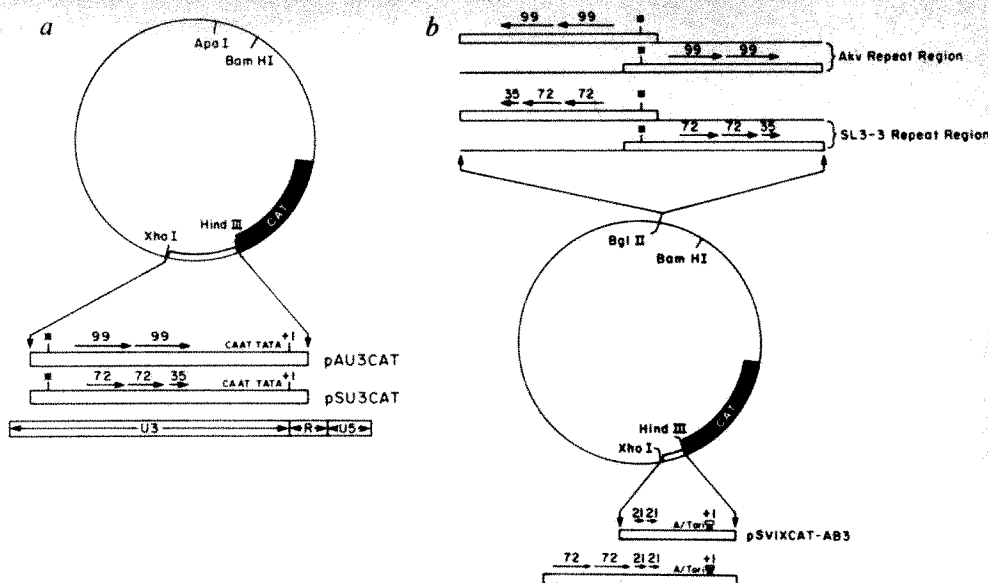
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Inoculation of susceptible strains of mice with the SL3-3 strain of murine leukaemia viruses induces T-cell lymphomas, whereas injection of the Akv strain does not¹⁻³. Recombinant viruses that contain the long terminal repeat (LTR) of the SL3-3 virus and the *gag*, *pol* and *env* genes of the Akv virus are also leukaemogenic⁴. The cell-type specificity of leukaemias induced by viruses containing different LTR sequences is due in part to the ability of the virus to replicate in the appropriate cellular environment⁴⁻⁷. One explanation of the role of the LTR in determination of both cell tropism and leukaemogenic potential is that the LTR encodes tissue-permissive transcriptional elements. We report here that there are differences in the transcriptional activity of the SL3-3 and Akv LTR sequences in different murine cell types, and that the sequences present in the LTR of SL3-3 exhibit significantly enhanced transcriptional activity in T cells compared with the corresponding region of the Akv LTR. The results suggest that transcriptional elements are primary determinants of cell tropism and of leukaemogenicity of these viruses.

To assess the ability of the Akv and SL3-3 LTR sequences to serve as promoter elements in different cellular environments, isogenic constructions⁸ were made in which the U3 regions of Akv (pAU3CAT) and SL3-3 (pSU3CAT) were placed 5' to the chloramphenicol acetyltransferase (CAT) gene⁹ (Fig. 1a). These

Fig. 1 Construction of plasmids used to assess promoter element function (a) and enhancer element activity (b) of murine LTR sequences. **a**, The plasmid subclones harbouring the 5'-LTR of Akv and SL3-3 (pLTR-A4 and pLTR-S3, respectively) were provided by J. Lenz⁴. The *Pst*I-*Ava*I fragment of the LTRs comprises the U3 and a small fraction of the R regions, extending from -443 to +31, relative to the viral RNA start site (designated as +1) for both viruses. This fragment was isolated from each LTR subclone where the natural *Ava*I site was converted to a *Hind*III site using *Hind*III synthetic linkers, and the natural *Pst*I site was converted to a blunt end. The plasmid that served as the recipient vector of the LTR fragments contains the entire CAT gene transcription unit⁹ (CAT coding sequences followed by the SV40 t-intron and polyadenylation signals) under the control of a SV40 early region promoter mutant that carries a deletion of nearly the entire 72-bp tandem repeat element (designated as pSVIXCAT, see below). The SV40 early region promoter, bounded by a synthetic *Xho*I site (5') and a natural *Hind*III site (3'), was removed from pSVIXCAT by successive digestion with *Xho*I, T4 DNA polymerase and *Hind*III enzymes in a fashion whereby the *Xho*I site was converted to a blunt end. The LTR fragments were ligated to the vector fragment containing the CAT gene transcription unit to generate plasmids in which the CAT gene is under the control of the transcriptional elements in the U3 region of Akv (pAU3CAT) or SL3-3 (pSU3CAT). The Akv and SL3-3 U3 regions are identical except for the presence of a single base insertion at nucleotide position 65 (designated by an asterisk) in SL3-3 relative to Akv, and the sequences within the repetitive elements (designated by arrows)⁴. The relative positions of CAAT, TATA and initiation (designated as +1) sequences are indicated. The location and boundary of LTR sequences used in pAU3CAT and pSU3CAT are shown relative to a typical retroviral LTR. **b**, To construct plasmids to assess enhancer element activity, a plasmid that carries the CAT gene under the control of the SV40 early region promoter (designated as pSV2CAT⁹) was digested with *Acl*I and *Sph*I to remove the majority of the 72-bp repeat region. The natural *Acl*I and *Sph*I sites were converted to *Xho*I sites using T4 DNA polymerase and a *Xho*I synthetic linker. Following digestion with *Xho*I and recircularization in the presence of DNA ligase, the resulting plasmid (pSVIXCAT) contains the CAT gene under the control of SV40 promoter sequences extending from -127 to +65 (relative to the 5'-most early start site)²⁹. The structure of this promoter deletion mutant is shown relative to the entire SV40 early region promoter found in pSV2CAT. The repetitive elements (designated by arrows), the origin of replication (ori), the TATA element (denoted as A/T) and early start site(s) (designated as +1) are illustrated. The SV40 promoter sequences present in pSVIXCAT are deficient in enhancer element activity; however, this activity can be restored by linkage of sequences containing enhancer activity to pSVIXCAT at a variety of sites relative to the SV40 early start site(s) (unpublished observations). For this study, a variant of SVIXCAT (designated as pSVIXCAT-AB3) was constructed in which the natural *Apa*I site⁴⁰ located greater than 2.1 kilobases from the SV40 early start site(s) was converted to a *Bgl*II site using T4 DNA polymerase and a synthetic *Bgl*II linker. The LTR subclones, pLTR-A4 and pLTR-S3, were digested with *Sau*3A and *Eco*RI, and a 620-bp *Sau*3A fragment containing 337 bp of U3 region sequences and 283 bp of *Sau*3A fragment D of pBR322 was isolated. The U3 region sequence present in the 620-bp *Sau*3A fragment consists of sequences extending from -443 to -106 (relative to the viral LTR start site in **a**), and contains the entire tandem repeat region of each virus. The 620-bp *Sau*3A fragment from each LTR subclone was inserted into pSVIXCAT-AB3 in either orientation at the unique *Bgl*II site. All manipulations were done according to standard recombinant DNA techniques⁸.



plasmids were introduced into cultured cells using either the calcium phosphate¹⁰ or the DEAE-dextran methods¹¹. After a transient expression period of 44–50 h, cell extracts were prepared, and the level of CAT enzymatic activity was measured⁹. Previous investigators have demonstrated that for constructions such as those used here, in which the sequences surrounding the RNA start sites are identical, and which differ only in sequences located remotely from the start site⁴, the level of indicator gene activity corresponds directly to the transcription rate^{12–15}. To determine the level of nonspecific and specific expression of the CAT gene in murine cells respectively, parallel transfection experiments were conducted in which cultured cells were exposed to plasmids containing either no eukaryotic promoter element (pSVOCAT)⁹ or the 3'-LTR of Rous sarcoma virus (pRSVCAT)¹⁶ located 5' to the CAT gene.

The level of CAT activity following transfection of these plasmids into murine fibroblast cells is shown in Fig. 2 and Table 1. In NIH 3T3 cells, the level of CAT gene expression of the plasmid containing the Akv LTR sequences is three times that of the plasmid containing the corresponding region of the SL3-3 virus. A similar result was obtained using mouse L cells, another murine fibroblast cell line. From these results, we conclude that in some murine fibroblasts the Akv LTR sequences are more potent than those of SL3-3 in promoting CAT gene expression.

A similar set of experiments was done using murine lymphoid cell lines. In marked contrast to the results observed using

fibroblasts, the level of CAT activity directed by the plasmid containing the SL3-3 LTR sequences was much greater than that directed by the plasmid containing the corresponding Akv sequences in all the T-cell lines tested (Table 1, Fig. 2b). The SL3-3/Akv ratio of transcriptional element activities was 0.3–0.6 in fibroblasts and 6–20 in T lymphocytes. The preferred expression in T-cell lines of the pSU3CAT plasmid relative to that of pAU3CAT is evidently not a consequence of virus infection because the SL3-3/Akv ratio of transcriptional element activity in SL3-3-infected L691 cells (designated as L691.SL3-3) is similar to that found in uninfected L691 cells. Preference for SL3-3 LTR sequences is also observed in primary cell preparations freshly eluted from the thymus of NFS/N mice (Table 1). Evidently, the transcriptional preference of SL3-3 LTR sequences in the established T-cell lines is a general property of T cells. The preferential expression of pSU3CAT is not observed on transfection of the murine B-cell line, M12 (Table 1), suggesting that preferential expression of pSU3CAT is not a general property of all lymphoid cells.

The only differences in LTR sequence between SL3-3 and Akv are located within the tandemly duplicated region located 221–443 base pairs (bp) from the RNA start site⁴, a region shown to include enhancer element functions of other murine retroviruses^{17–28}. This observation raises the possibility that sequences located far from the start site function as primary determinants of the efficiency of promoter element utilization in different cell types. To test this possibility, we measured the ability of the

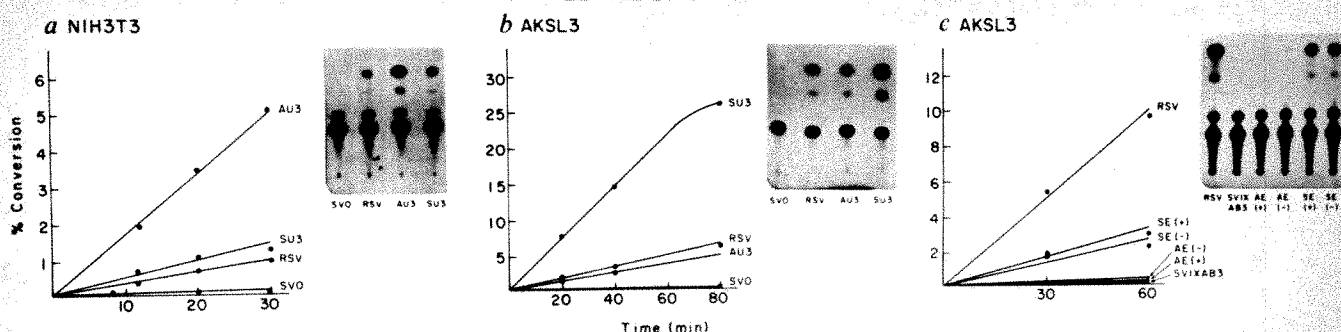


Fig. 2 CAT activity directed by viral LTR sequences in different murine cells. Representative CAT assays for plasmids containing either the Akv U3 region, abbreviated AU3; the SL3-3 U3 region, abbreviated SU3; the 3'-LTR of Rous sarcoma virus, abbreviated RSV; or no eukaryotic promoter element, abbreviated SVO, linked 5' to the CAT gene transcription unit transfected in parallel into cultured cells are shown for NIH 3T3 fibroblasts (a) and AKSL3 T cells (b). c, Representative CAT assay of enhancer element function for plasmids containing either the SV40 promoter sequences lacking enhancer linked sequences, abbreviated SVIX-AB3; the Akv repeat region inserted at the *Bgl*II site 2 kilobases away from the SV40 early start site(s) in the same and opposite transcriptional sense as CAT gene transcription, abbreviated AE(+) and AE(-), respectively; the SL3-3 repeat region inserted at the *Bgl*II site and in the same and opposite transcriptional sense as CAT gene transcription, abbreviated SE(+) and SE(-), respectively; and the 3' LTR of Rous sarcoma virus, abbreviated RSV, linked to the CAT gene and transfected in parallel into cultured cells, is illustrated for AKSL3 T cells. Results are expressed as percentage conversion of ^{14} C-chloramphenicol to ^{14} C-chloramphenicol acetate by extracts containing 400 μ g protein in a and b and extracts containing 600 μ g protein in c. The insets show typical autoradiograms of CAT assays at 30 min (a), 80 min (b) and 30 min (c). The upper two spots correspond to the two isomers of ^{14} C-chloramphenicol monoacetate while the lower spot is the unreacted substrate, ^{14} C-chloramphenicol.

Methods: Each murine cell line was transfected with an equivalent amount of each plasmid DNA per transfection cocktail. The amount of DNA used for each experiment was within the linear range of optimal CAT activity (data not shown). The NIH 3T3 cell transfections were carried out as described previously¹⁰. The L cell and lymphoid cell transfections were done as described previously¹¹. After a transient expression period (44 h in promoter studies as in a and b; 50 h in enhancer studies as in c), the cells were washed with cold phosphate-buffered saline and resuspended in 200 μ l of 0.25 M Tris-HCl pH 7.9. Cell extracts were prepared by three cycles of freeze (-70°C)-thaw (37°C) treatment and centrifugation. *In vitro* CAT enzymatic assays were performed according to published procedures⁹ except that a final concentration of 2.4 mM acetyl coenzyme A was used in each extract assay. Analysis and quantitation of *in vitro* enzymatic assay was done according to previously described procedures⁹.

portion of the U3 region harbouring the tandemly repeated element of SL3-3 and Akv LTR sequences to function as transcriptional enhancer elements in murine T cells. The portion of the simian virus 40 (SV40) early region promoter that governs start site specificity^{29,30} was positioned 5' to the CAT gene (designated as pSVIXCAT-AB3), and the region harbouring the tandem repeat element of either SL3-3 or Akv was linked in *cis*, in both orientations, at a site about 2 kilobases from the SV40 early start sites (Fig. 1b). These plasmids were transfected into murine T cells (the AKSL3 cell line), and the levels of CAT activity were determined relative to pRSVCAT and pSVIXCAT-AB3 (Fig. 2c, Table 2). The level of CAT activity directed by the plasmid containing the SL3-3 repeat region was more than 30 times that of the pSVIXCAT-AB3 plasmid that lacks murine LTR sequences. In contrast to the marked increase in CAT activity observed for the plasmids containing the SL3-3 sequences, the level of CAT activity for those plasmids containing the Akv tandem repeat region sequences was increased only twofold relative to pSVIXCAT-AB3. Similar results were obtained using another murine T-cell line, the SL3B cell line (data not shown).

These experiments demonstrate that in T cells the region harbouring a tandemly repeated element of the SL3-3 LTR behaves as a transcriptional enhancer element^{31,32}; that is, it potentiates increased activity from a remotely located hetero-specific promoter element in an orientation-independent manner. Thus, the enhanced transcriptional activity of the SL3-3 LTR sequences relative to Akv sequences in murine T cells can be attributed to differences in enhancer element function encoded by sequences remotely located in the U3 region.

The increased rate of CAT gene transcription directed by the SL3-3 LTR element in T cells, relative to that of Akv sequences, permits a natural explanation of the T-cell tropism and leukaemogenic potential of the SL3-3 LTR-containing viruses. Replication of retroviruses proceeds via transcription from integrated proviruses. Tissue-specific differences in the rate of transcription of the magnitude observed here, 10–60-fold, may have a profound effect on the extent of viral replication, as such differences might be expected to be magnified as a power function on successive rounds of replication. Consonant with this

model, recent studies indicate that a correlation exists between the cell specificity of viral replication and the type of leukaemia induced by an isogenic set of viruses that differ only in the LTR regions^{3–7}. Thus, it is likely that viral replication, via transcription, in the appropriate cellular context may have a

Table 1 Relative activity of murine LTR sequences as promoter elements in various cell types

Cell type	Promoter element				SL3-3/Akv U3 region activity ratio
	None	RSV 3' LTR	Akv U3 region	SL3-3 U3 region	
Fibroblasts					
NIH 3T3	0.02	1.00	4.94	1.52	0.31
L	0.05	1.00	5.96	3.74	0.62
Lymphoid cells*					
AKSL3	0.01	1.00	0.76	4.50	5.94
SL3B	0.01	1.00	0.51	10.46	20.51
L691	0.04	1.00	0.64	4.09	6.39
L691.SL3-3	0.01	1.00	0.24	1.73	7.20
NFS/N thymocytes	0.85	1.00	0.92	13.57	14.75
M12	0.01	1.00	2.76	1.75	0.64

The CAT activity of each plasmid is normalized relative to the absolute activity of the 3'-LTR of RSV in pRSVCAT within each cell line. Absolute activity for each plasmid in every cell line was calculated from the slope of the line in the initial velocity of CAT enzymatic assay for cell extracts containing 400 μ g of protein as depicted in Fig. 2. The absolute activity in any given cell line transfected with each plasmid is expressed in terms of % conversion per h in the *in vitro* assays, and may be obtained quantitatively by multiplying the relative value in the table by the absolute activity value of the same cell line transfected with pRSVCAT. The absolute activity of pRSVCAT in each cell line is given in parentheses: NIH 3T3 ($2.06\% \text{ h}^{-1}$), L ($0.64\% \text{ h}^{-1}$), M12 ($2.5\% \text{ h}^{-1}$), AKSL3 ($4.87\% \text{ h}^{-1}$), SL3B ($2.04\% \text{ h}^{-1}$), L691 ($0.84\% \text{ h}^{-1}$), L691.SL3-3 ($2.37\% \text{ h}^{-1}$) and NFS/N thymocytes ($0.03\% \text{ h}^{-1}$). Results shown here reflect an average of at least two independent transfection experiments, and CAT assays performed using extracts containing 400 μ g of protein. The range of activities around a particular value varied by not more than 30% of that value.

* Several T-cell lines established from murine T lymphomas were used as recipients and include: AKSL3, a cell line established from a spontaneous tumour of an AKR mouse (the cell line from which the SL3-3 virus was isolated)³⁶; SL3B, a cell line derived from a thymic T-cell tumour of an AKR mouse that had been injected with the SL3 virus³⁷; the uninfected murine T-cell line, L691 (ref. 38); L691.SL3-3, L691 cells infected *in vitro* with SL3-3 virus; and freshly eluted primary thymus cells isolated from NFS/N mice³⁹. The murine B-cell line, M12, was also used as a recipient.

Table 2 Relative activity of murine LTR sequences as enhancer elements in the murine T-cell line AKSL3

Promoter element	Viral repeat region and orientation	Relative activity	Relative-fold enhancement
RSV 3'-LTR		1.000	—
SV40 early region mutant	None	0.01	1.00
SV40 early region mutant	Akv, sense	0.01	1.00
SV40 early region mutant	Akv, antisense	0.02	2.00
SV40 early region mutant	SL3-3, sense	0.37	37.00
SV40 early region mutant	SL3-3, antisense	0.34	34.00

The CAT activity of each plasmid is normalized relative to the absolute activity of the 3'-LTR in pRSVcat within the T-cell line AKSL3. Absolute activity was determined as described in Table 1. Results shown here are an average of three transfection experiments and CAT assays were performed using extracts containing 600 µg of protein. The range of activities around a particular value varies by not more than 30% of that value. Fold-enhancement for plasmids containing viral repeat regions was determined relative to pSVXCAT-AB3, the plasmid lacking a viral repeat region.

major role in the induction of a tissue-specific disease by these viruses. Finally, recent studies suggest that murine leukaemia viruses may induce disease by proviral integration adjacent to common loci^{28,33-35}, some of which harbour known oncogenes^{28,34}. Analysis of these integration sites indicates that these loci may be transcriptionally activated by the adjacent viral LTR enhancer element^{28,33-35}. We speculate that an additional, if not simultaneous, characteristic of the LTR of a T-cell leukaemogenic virus is to provide strong enhancer element function sufficient to transcriptionally activate adjacent cellular loci.

We thank Corri Gorman and Bruce Howard for gifts of plasmids, Ester Hays for gifts of AKR T-cell lines, Philip Leder for the M12 cell line, and Jack Lenz, Joseph Sodroski, Craig Rosen and Miles Cloyd for helpful discussions. We thank Ms Donna Artz for help in preparation of this manuscript. This work was supported by NCI grant CA19341.

Received 27 July; accepted 6 September 1984.

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Transferrin receptor on endothelium of brain capillaries

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The blood/brain barrier prevents the passive diffusion of proteins and metabolites from cerebral blood vessels into tissue spaces around neuronal and glial cells. To provide nutrients for these cells, transport mechanisms must exist and indeed have been demonstrated for metabolites¹. We now show that monoclonal antibodies against rat and human transferrin receptors label blood capillaries in the brain but not in other tissues. In the rat this labelling occurs after injection of antibody into the blood, thus the receptors seem to be accessible at the endothelial surface. It is possible that transferrin receptors are expressed on these cells to allow transport of transferrin (and thus iron) into brain tissues.

The MRC OX-26 mouse IgG2a monoclonal antibody is specific for transferrin receptors on the basis that it binds to a detergent-solubilized molecule that can bind ¹²⁵I-transferrin and precipitates a dimer of glycoproteins of apparent molecular weight ~95,000 (ref. 2). Cryostat sections were cut from various rat tissues and stained with MRC OX-26 antibody, followed by peroxidase-conjugated rabbit F(ab')₂ anti-mouse IgG antibody³. One notable reaction occurred with what appeared to be capillaries in the brain; this is shown in Fig. 1a for a 5-µm section from the cerebellum of rat brain and in Fig. 1b for a 30-µm section. The thicker section clearly shows the branched structures, and similar patterns were seen in sections from the brain cortex and spinal cord. Sections from other rat tissues including thymus, lymph node, spleen, heart, kidney, liver, pancreas and small intestine were also stained with OX-26 antibody² but labelling of capillaries was not observed in these sections. In the sections of brain tissue, scattered cells having the morphology of neurones were also stained (for example, see Fig. 1a).

To test whether the antigen recognized by the MRC OX-26 antibody was accessible at the endothelial surface, 2.5 mg of MRC OX-26 IgG was injected intravenously (i.v.) into 4-week-old PVG/c rats and control animals were injected with an equal amount of W6/32 antibody (an IgG2a specific for human histocompatibility locus antigen) or normal mouse IgG. The rats were killed 20 min or 16 h after antibody injection and cryostat sections were cut from their brains and stained with peroxidase-conjugated rabbit anti-mouse IgG antibody. Figure 1c shows clear labelling of the capillaries in a section from an animal given OX-26 antibody 20 min previously, but not in a section from an animal injected with control antibodies. Labelling was still evident 16 h after the injection of antibody, but was weaker than at the earlier time (not shown).

The above results suggest that transferrin receptors are expressed at the luminal surface of the endothelium of rat brain capillaries. Alternatively, the staining could be due to a spurious cross-reaction with an unrelated molecule; this possibility would be excluded if similar reactions were seen with other monoclonal antibodies against transferrin receptors. Several antibodies that react with human transferrin receptors have been described and the antibodies B3/25 (ref. 4), T56/14 (ref. 5) and OKT9 (ref. 6) unequivocally have this specificity. When used to stain sections from two human brains, these antibodies stained the capillaries in both cases. In one case, sections from somatic and

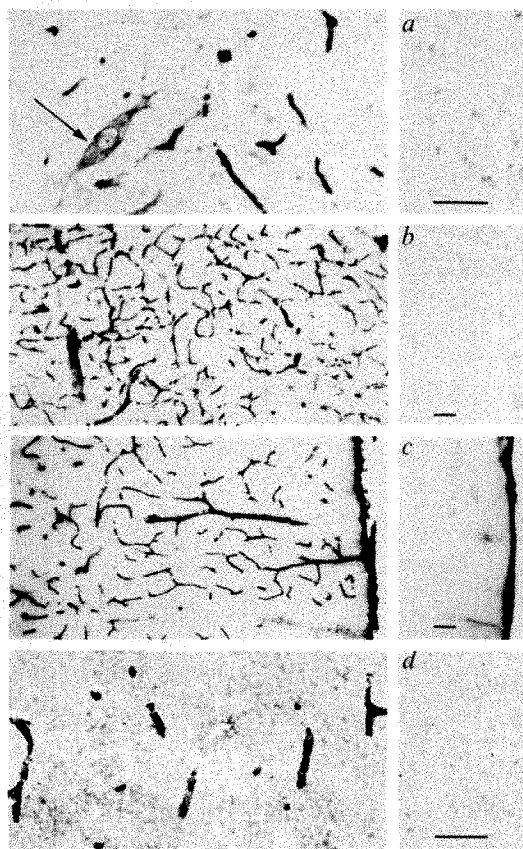


Fig. 1 Staining of sections from rat and human brain with anti-transferrin receptor monoclonal antibodies. *a, b*, Labelling by peroxidase immunohistochemistry of 5- μ m and 30- μ m cryostat sections cut from the cerebellum of 12-week-old PVG/c rats. The sections were cut, fixed and stained with MRC OX-26 monoclonal antibody, followed by peroxidase-conjugated rabbit F(ab')₂ anti-mouse IgG antibody exactly as described in ref. 3. The monoclonal antibody was used as a tissue culture supernatant from OX-26 hybridoma cells. The control shown at the right of each section comprised an adjacent section stained with an antibody that did not react with rat tissues (IgG1 or IgG2a control antibodies were equally unreactive). The arrow in *a* indicates a labelled neurone. *c, d*, A 25- μ m section from the cerebral cortex of a rat which had been given 2.5 mg of OX-26 IgG i.v. 20 min before killing. This section was stained with peroxidase-conjugated rabbit F(ab')₂ anti-mouse IgG antibody without any first incubation with monoclonal antibody. The control is a section from a rat given 2.5 mg of W6/32 antibody (in ascites fluid) 20 min before killing. *d*, A 9- μ m cryostat section from the cerebellum of a 25-yr-old female human; the tissue was obtained during autopsy (~7 h after death). The section was stained with B3/25 monoclonal antibody and the bound antibody detected by alkaline phosphatase immunohistochemistry, in which the enzyme was cross-linked to the monoclonal antibody by a bridge of rabbit anti-mouse IgG antibody and mouse monoclonal anti-alkaline phosphatase antibody⁹. The sections in *a* and *d* are at a magnification 2.5 times greater than that of *b* and *c*, and the bars in the control sections represent 50 μ m.

visual cortex, basal ganglia, cerebellum, mid-brain and spinal cord were stained and labelling of capillaries was evident in all these areas. Figure 1*d* shows a typical result for antibody B3/25 staining a section of cerebellum. In addition, staining for factor VIII, an established marker for capillaries⁷, was done using the F8.44.20 monoclonal antibody⁸ and the patterns of reaction (not shown) were indistinguishable from those in Fig. 1*d*. In other human tissues, the capillaries were clearly labelled with the anti-factor VIII antibody, but staining of capillaries was not observed with the anti-transferrin receptor antibodies⁵. Labelling of human neurones is not evident in Fig. 1*d*, but scattered neuronal staining was seen in sections of human as well as in rat brain.

The reaction of blood capillaries in human and rat brain with four different monoclonal antibodies suggests strongly that transferrin receptors are expressed on the capillaries, and the results in rat show that these are located at the luminal surface. It is possible that these molecules are involved in the transport of transferrin across the blood/brain barrier.

We thank Margaret Hughes for technical assistance, Stan Buckingham for photography, Dr T. Powell for advice on obtaining rat brain material, Dr A. N. Barclay for peroxidase-conjugated rabbit F(ab')₂ anti-mouse IgG antibody and Dr I. S. Trowbridge for antibodies B3/25 and T56/14. This work was supported in part by the Leukemia Research Fund and the Wellcome Trust. W.A.J. is an Exhibition of 1851 Scholar and acknowledges a BCHCRF traineeship award.

Received 11 May; accepted 5 September 1984.

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Expression of rabies virus glycoprotein from a recombinant vaccinia virus

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Rabies is one of the oldest diseases known to man, but its successful control has remained elusive. Although effective vaccines of tissue culture origin against rabies do exist¹, such preparations are expensive. Live vaccinia virus (VV) recombinants expressing influenza or hepatitis B antigens have recently been used to immunize against these diseases²⁻⁴. We have now used this approach to produce a novel rabies vaccine. We first altered the rabies glycoprotein cDNA⁵ by site-directed mutagenesis and removed the poly(dG) tail. We then aligned the modified cDNA with an early VV promoter sequence inserted within a cloned copy of the vaccinia thymidine kinase gene and transfected this plasmid into VV-infected cells. Recombination between the virus and the plasmid resulted in a recombinant virus harbouring the rabies glycoprotein cDNA. Inoculation of rabbits with the live recombinant virus induced high titres of rabies virus-neutralizing antibodies, and scarification with the recombinant VV protected mice against challenge with street rabies virus.

The rabies virus contains five virus-encoded proteins, only one of which, the glycoprotein, traverses the lipid bilayer envelope. The glycoprotein spike is thus the sole viral component capable of reacting with virus-neutralizing antibodies and eliciting their production. The full-length coding sequence of the ERA strain rabies glycoprotein messenger RNA has been isolated⁵ and its sequence expressed in a bacterial host⁶. Parallel experiments have been described⁷ using the related CVS strain of rabies, but no protection against rabies using the bacterially synthesized material has yet been reported. These data suggest that post-translational modification and/or presentation of the glycoprotein may be important parameters in the use of this antigen in protection against rabies. We have explored the use

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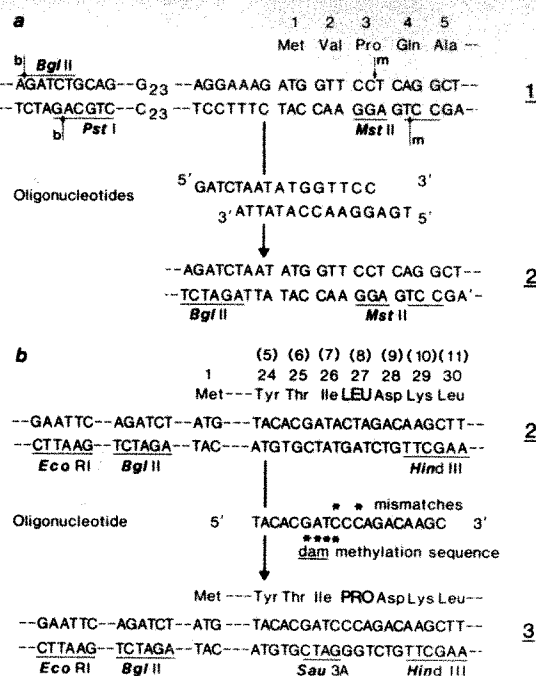
Fig. 1 Restructuring of the rabies glycoprotein cDNA. *a*, The cDNA segment between the *Bgl*II/*Pst*I site at one extremity and a downstream *Mst*II site is replaced by a synthetic double-stranded oligonucleotide adaptor to delete the upstream poly(G)/poly(C) sequence. In *b* the codon for amino acid 8 in the cDNA sequence is changed from CTA to CCA using oligonucleotide-directed mutagenesis. Amino acids of the primary translation are numbered; numbers in parentheses give the amino acid positions in the mature glycoprotein molecule after removal of the N-terminal signal sequence. The precise cleavage sites for *Mst*II and *Bgl*II, respectively, are indicated by 'm' and 'n'. Asterisks indicate mismatches and the *dam* methylation sequence.

Methods: Restructuring the initiation region: In plasmid ptg150(1), the rabies glycoprotein cDNA of Anilionis *et al.*⁵ was introduced, using octameric *Pst*I-*Bgl*II oligonucleotide adaptors¹⁶, into a unique *Bgl*II site inserted at the *Hind*III site of plasmid pBR327 (R.L., unpublished data). This plasmid was digested partially with *Bgl*II and completely with *Mst*II, and the appropriate linear fragment isolated by electrophoresis on a low gelling temperature agarose gel. Oligonucleotides 5'-dGATCTAATATGGTTCC-3' and 5'-dTGAGGAACCATATTA-3' were synthesized using a previously described technique¹⁷. These were hybridized together and ligated between the *Bgl*II and *Mst*II extremities of the purified linear plasmid, permitting recircularization and generating plasmid ptg155(2). Correction of triplet 8: The oligonucleotide 5'-dTACACGATCCCAGACAAGC-3' for localized mutagenesis¹⁸ contains two mismatches with the original sequence: a T to C transition correcting the leucine triplet, CTA, to proline, CCA, and an adjacent A to C transversion in the sequence GATA. This generates a *dam* methylation sequence GATC which favours incorporation of the oligonucleotide sequence by selective mismatch correction *in vivo* following primed repair and transfection (see refs 19, 20 for theoretical background to this strategy). The *dam* methylation sequence is itself a restriction recognition site for the enzyme *Sau*3A and permitted rapid identification of correct constructs from digestion profiles of replicative form DNAs. Fragment exchange generated the final reconstructed plasmid ptg155-PRO. Note that the *Eco*RI/*Hind*III fragment was cloned in the reverse orientation of that presented here: the lower strand (complementary to the oligonucleotide) was present in the parental single-stranded M13 bacteriophage recombinant. Hybrid plasmid assembly: Miniplasmid ptg1H was constructed starting from plasmid pML2²¹ (a spontaneous deletion derivative of vector plasmid pBR322 lacking the segment between nucleotides 1,098 and 2,490: probable sequence at deletion boundaries AGCTTCACGGCCAGC, R.L., unpublished results). The *Pst*I recognition sequence was removed by inserting a 692 base pair (bp) *Aha*III-*Aha*III fragment of pUC8²² between two *Aha*III sites of pML2 (removing 19 bp) and the linker-tailing method²³ was used to insert a *Hind*III linker between the *Nru*I and *S*₁-treated *Eco*RI sites of this plasmid (ptg1H). The 4.6 kb *Hin*-J fragment of VV DNA bearing the *tk* gene¹¹ was inserted at the unique *Hind*III site to generate ptg1H-TK. The VV *Sal*-S fragment, carrying the 7.5K promoter²⁴, was isolated in an M13 vector²⁵ as a (shotgun) *Sal*I clone of VV DNA (M13tg*Sal*-S). Here a *Sca*I site lies 33 nucleotides downstream of the transcription start point and 18 nucleotides upstream from the putative initiation codon for the 7.5K gene²⁴. This clone was truncated using the linker-tailing protocol²³ to insert a *Bgl*II linker oligonucleotide 5'-dCAGATCTG-3' between the *Sca*I site and the M13 vector *Bam*HI site after filling the extremity generated by *Bam*HI digestion by DNA polymerase (Klenow fragment) treatment. This eliminates both the *Sca*I site and the downstream *Sal*I (*Acc*I) site, the upstream site becoming unique (M13tg7.5K). The promoter fragment (~270 bp) was excised with *Acc*I and *Eco*RI and ligated between the unique *Cl*A and *Eco*RI sites (see ref. 26) within the *tk* gene present in ptg1H-TK and generating ptg1H-TK-7.5K, deleting 33 bp (see ref. 27 for an alternative cloning strategy). The *Bgl*II-*Bgl*II fragment from ptg155-PRO was subsequently inserted into the unique *Bam*HI site of ptg1H-TK-P7.5K to generate pVVTGgRAB.

of a VV vector to express the rabies glycoprotein coding sequence.

Expression of exogenous protein coding sequences in VV involves two steps. First, the exogenous coding sequence is aligned with a functional VV promoter and inserted *in vitro* at a site within a (non-essential) segment of VV DNA cloned into a suitable bacterial plasmid replicon. Second, the flanking VV sequences now permit homologous recombination *in vivo* between the plasmid and the viral genome. Double reciprocal recombination results in transfer of the DNA insert from the plasmid to the viral genome, wherein it is propagated and expressed^{2,4,8,9}.

The ERA rabies glycoprotein coding sequence⁵ carries two impediments to its successful translation and expression as an antigen. First, the ATG corresponding to the initiation codon of the mature message lies adjacent to a poly(dG) sequence introduced by the cDNA cloning procedure. This was eliminated by replacing the appropriate cDNA segment by a synthetic double-stranded adaptor, deleting the G/C tail and reconstructing the initiation codon region (plasmid ptg155; Figs 1a, 2). Second, the eighth amino acid of the mature glycoprotein (lacking its signal sequence) of rabies virus ERA and CVS strains is proline^{7,10}, whereas in the cDNA isolated by Anilionis *et al.*⁵, the triplet at this position codes for leucine. The appropriate segment of glycoprotein cDNA was subcloned into a single-stranded bacteriophage vector and oligonucleotide-directed mutagenesis was used to correct the triplet CTA (Leu) to CCA (Pro) (Fig. 1b).



A *Hind*III fragment (*Hin*-J) of the VV genome containing a complete thymidine kinase (*tk*) gene has been used previously to permit recombinational exchange of inserted DNA into the VV genome^{2,3,9}. Transfer of an insert in the *tk* gene to the VV genome creates an easily recognized TK-deficient (TK⁻) virus (Fig. 1). Next, a VV promoter was required to drive the expression of the inserted rabies glycoprotein coding sequence. The promoter of the early VV gene encoding a 7,500 molecular weight (MW, 7.5K) protein has previously been used successfully for a similar purpose^{2,3}, and we inserted this segment into the cloned *tk* gene (Fig. 1 legend). The corrected rabies glycoprotein cDNA was introduced downstream of the (7.5 K) promoter to generate pVVTGgRAB (Fig. 1 legend). This plasmid carries all the elements necessary for transfer of the rabies glycoprotein coding sequence to the VV genome and its subsequent expression.

The strategy devised by Smith *et al.*² relies on *in vivo* exchange between a plasmid bearing an insert within the VV *tk* gene and wild-type viral genome to inactivate the virus-borne *tk* gene. TK⁻ viruses may be selected by plating on a TK⁻ cell line in the presence of 5-bromodeoxyuridine (BrdUrd)⁹. TK phosphorylates BrdUrd to the 5'-monophosphate, which is converted subsequently to the triphosphate. This compound is an analogue of dTTP; its incorporation into DNA prevents correct development of the virus. A TK⁻ virus can, however, replicate its DNA normally and give rise to visible viral plaques on a monolayer of an appropriate TK⁻ cell line.

VV propagates in the cytoplasm of infected cells rather than

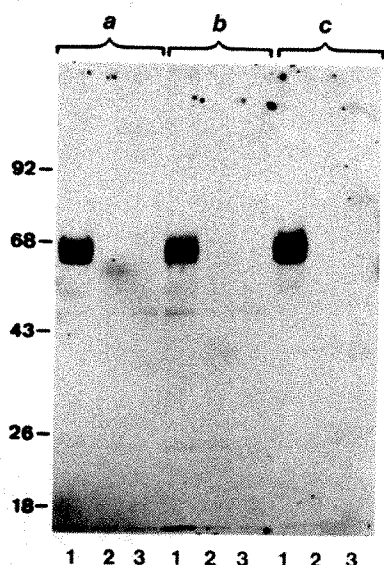


Fig. 2 Immunoprecipitation of proteins synthesized after infection by the VV-rabies recombinant. *a*, Polyclonal antiserum R215 (Wistar) raised against the purified glycoprotein was used; *b*, *c*, identical results were obtained with two monoclonal virus-neutralizing antibodies (509-6 (*b*) and 101-1 (*c*); Wistar) which identify different epitopes on the rabies glycoprotein. The cell monolayers were infected with: 1, recombinant VVTGgRAB-26D3; 2, wild-type VV; 3, no virus. Numbers on the left-hand side represent molecular weight standards ($\times 10^{-3}$).

Methods: Semi-confluent monolayers of LTK⁻ cells ($\sim 10^6$ per plate) were adsorbed with VVTGgRAB-26D3 (50 PFU per cell) for 1 h at room temperature, methionine-free culture medium was added and after 30 min each plate was supplemented with 50 μ Ci of ^{35} S-L-methionine (1,265 Ci mmol⁻¹; Amersham) and incubation continued at 37 °C for 4 h. Cells were collected and resuspended into immunoprecipitation buffer containing protease inhibitors. After sonication and clarification, proteins bound by anti-rabies antisera were recovered by affinity chromatography on protein-A-Sepharose and displayed by gel electrophoresis and fluorography by a modification of a previously described protocol²⁸.

in the nucleus. It is thus unable to take advantage of the host DNA replication and transcription machineries and instead carries in the virion those components necessary for the onset of viral gene expression. For the purpose of generating recombinants, it is necessary simultaneously to infect cells with live VV and introduce the cloned DNA segment of interest by calcium-mediated transfection. However, because the generation of recombinants is limited to those few cells competent for DNA transfection, we used an indirect 'congruence' strategy to reduce the background of non-recombinant parental viruses. This was achieved by using as live infecting virus a ts (temperature-sensitive) mutant of vaccinia unable to propagate at the non-permissive temperature¹¹. When cells infected with a ts mutant in non-permissive conditions are transfected with wild-type virus DNA, viral multiplication occurs only in those cells competent for transfection.

Monolayers of primary chick embryo fibroblast (CEF) cells were infected with VV-Copenhagen ts26 (0.1 plaque-forming units, PFU, per cell), incubated at the permissive temperature (33 °C) for 2 h, then transfected with a calcium phosphate coprecipitate of VV-Copenhagen wild-type DNA ($\sim 0.5 \mu$ g per 10^6 cells) and the recombinant plasmid pVVTGgRAB (3.0 μ g per 10^6 cells). After incubation for 2 h at the non-permissive temperature (39.5 °C), the cells were rinsed and incubated for 48 h at 39.5 °C. Dilutions of ts⁺ virus emerging were reinfected onto a monolayer of mouse LTK⁻ cells at 37 °C and incubated in the presence of BrdUrd (100 μ g ml⁻¹). TK⁻ plaques were obtained from cells that had received the recombinant plasmid, whereas in control cultures without the plasmid no plaques were visible (data not shown).

Table 1 The potential of the recombinant virus to induce rabies-neutralizing antibodies

Post-vaccination day	VVTGgRAB-26D3	Vaccinia wild-type
0	<10	<10
11	10,000	<10
14	>30,000	<10

Rabbits were injected intradermally with 2×10^8 PFU of VVTGgRAB-26D3 virus or with wild-type VV. Inoculations were made in three points on the back. On days 0, 11 and 14, rabbits were bled and sera tested for the presence of rabies virus-neutralizing antibody by a modified rapid fluorescent focus inhibition test¹⁵. Rabies virus was preincubated for 1 h with dilutions of the rabbit antisera and adsorbed to BHK-21 cells. Productively-infected cells were stained after 24 h using a direct immunofluorescence technique. Titres given as the highest dilution at which 50% inhibition of virus infection was observed.

Correct double reciprocal recombination between the hybrid vaccinia-rabies plasmid and the VV genome exchanges the viral *tk* gene for the insert-bearing *tk* gene present on the plasmid. We confirmed the presence of the rabies glycoprotein cDNA by digesting DNA purified from TK⁻ virus with *Hind*III. The 4.6 kilobase (kb) *Hin*-J fragment was absent from these viruses, as we expected, and two new fragments of ~ 1.1 and 5.5 kb revealed the presence of an insert containing an internal *Hind*III site. Southern blotting confirmed that the insert hybridized with rabies glycoprotein cDNA (data not shown) and one recombinant, VVTGgRAB-26D3, was retained. This virus was assessed for its ability to direct the synthesis of rabies glycoprotein in tissue culture cells.

First, monolayers of LTK⁻ cells infected with VVTGgRAB-26D3 were examined for *in situ* immunofluorescence using virus-neutralizing antibodies. Most cells exhibited strong fluorescence associated with the cytoplasmic membrane, whereas non-infected cells and cells infected with the non-recombinant virus showed no fluorescence (data not shown). We infected monolayers of semi-confluent LTK⁻ cells with VVTGgRAB-26D3; polyclonal rabies antiserum as well as two different virus-neutralizing monoclonal antibodies precipitated a protein migrating as a diffuse band of MW 66,000 (Fig. 2). This molecular weight corresponds to the size of ERA virus glycoprotein¹². Furthermore, sera from rabbits immunized with VVTGgRAB-26D3 recognized and bound radiolabelled authentic viral glycoprotein (data not shown). Sera from animals immunized with the non-recombinant VV gave no such reaction. We subsequently examined the potential of the recombinant virus to induce rabies-neutralizing antibodies. Even at the highest dilution of the 14-day antisera, complete neutralization of the virus was achieved.

These results demonstrate that the recombinant VVTGgRAB-26D3 not only induces the production of antibodies reacting with rabies virus, but also that antisera from immunized animals are capable of inactivating rabies virus *in vitro*. This encouraged direct protection testing. Mice immunized by intradermal inoculation of VVTGgRAB-26D3 virus resisted challenge with large

Table 2 Resistance of immunized mice to rabies virus

Immunizing agent	% Protection
None	0
Vaccinia wild-type virus	0
Recombinant virus VVTGgRAB-26D3	100

Female mice (ICR strain) were infected intradermally by scarification with $\sim 5 \times 10^7$ PFU of VVTGgRAB-26D3 or wild-type VV. Four skin scratches were made on the base of the tail using a hypodermic needle and virus suspension rubbed over the abrasion. On day 15 all vaccinated and control animals were infected by intracerebral inoculation with CDC strain tissue-culture adapted street virus (2,400 mouse LD₅₀ units). Test animals were monitored for 2 months post-challenge: unprotected mice succumbed after 8–10 days.

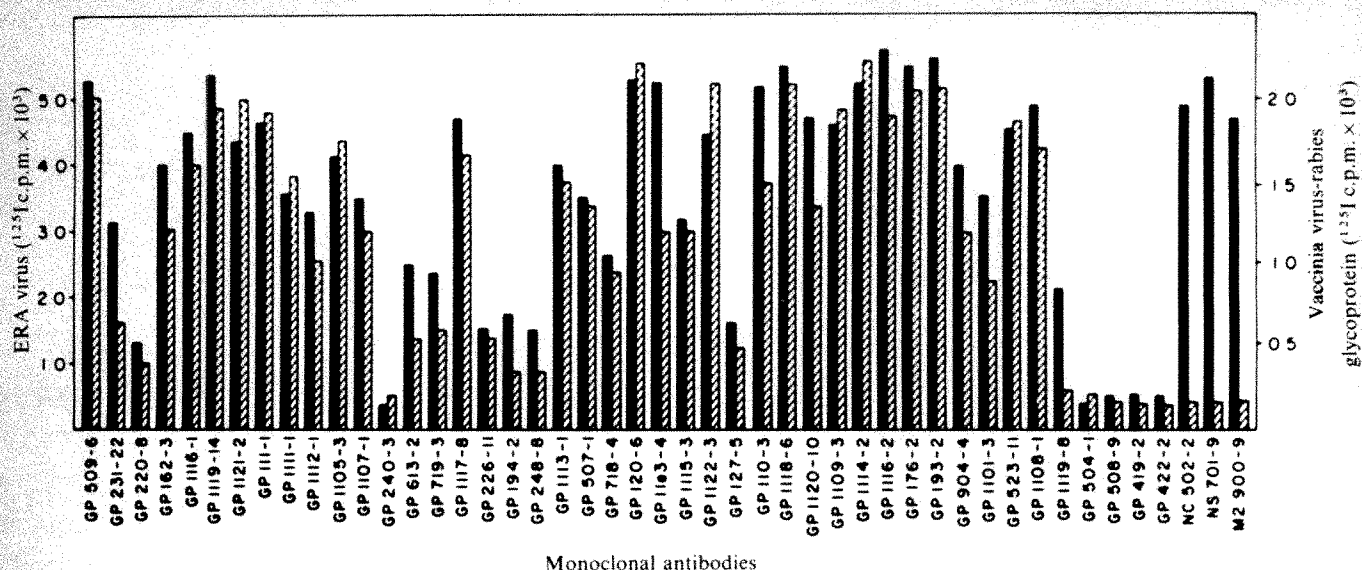


Fig. 3 Comparative binding of VVTGgRAB and ERA virus antigens with a panel of monoclonal antibodies. Solid bars, ERA virus; cross-hatched bars, VVTGgRAB virus.

Methods: Antigens (100 ng) were dried on microtitre plates and treated for 30 min with phosphate-buffered saline (PBS) containing 10% γ -globulin-free horse serum (Gibco). After draining, monoclonal antibody (1:1,000 dilution of ascites fluid; 25 μ l) was added, incubated for 1 h at 37 °C and washed with PBS. Each well then received 25 μ l of ^{125} I-labelled goat anti-mouse antibodies (30,000 c.p.m., specific activity, 0.5 mCi mg $^{-1}$). After further incubation (37 °C, 1 h) and washing with PBS, the bottom of each well was cut out and radioactivity determined.

doses of street rabies virus, whereas mice similarly immunized with wild-type VV alone were not protected (Table 2).

To assess the authenticity of the recombinant rabies glycoprotein, reactivity with a panel of monoclonal antibodies directed against rabies glycoprotein and other viral proteins (N, NS and M) was examined. The binding activity of the recombinant glycoprotein with 44 anti-glycoprotein monoclonal antibodies was almost identical to that observed with purified ERA rabies virus, whereas only the ERA virus reacted with anti-N, -NS and -M antibodies (Fig. 3). This demonstrates that the rabies glycoprotein produced by VVTGgRAB virus-infected cells is qualitatively indistinguishable from the native glycoprotein of ERA virus.

Vaccinia virus has been used extensively as a live vaccine to control and eradicate smallpox (see ref. 13 for review); it has been developed as a cloning and expression vehicle for hepatitis B, influenza and herpes antigens and protection has been achieved by vaccination with appropriate influenza- and herpes-VV recombinants^{2-4,14}. We demonstrate here that live VV expressing the rabies glycoprotein is capable of conferring protection against experimental rabies infection. Attenuated viruses such as VV are particularly appropriate vehicles for vaccine production: their preparation and administration can avoid costly procedures involving propagation of the pathogenic agent on cultured mammalian cells and subsequent toxicity testing.

We thank A. Kirn and D. Nayak for helpful discussions and P. Chambon, E. Eisenmann and P. Kourilsky for encouragement and critical reading of the manuscript, A. Balland for preparing the synthetic oligonucleotides used in this work, D. Villeval and F. Jaeger for verifying constructs by sequencing and E. Chambon and F. Daul for assistance in preparing this manuscript. This study was supported in part by NIAID grant AI-09706.

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Molecular cloning and characterization of the HTLV-III virus associated with AIDS

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We recently reported the isolation and characterization of a novel human T-lymphotropic retrovirus, HTLV-III, in patients with acquired immune deficiency syndrome (AIDS) and in those at risk for the disease¹⁻⁴. After extensive sero-epidemiological studies^{3,5,6}, together with numerous virus isolations from these patients^{1,7}, we concluded that HTLV-III is the causative agent of AIDS. Here we report the molecular cloning and characterization of two highly related but distinct forms of the HTLV-III genome. The viral genome is ~10 kilobases long and is detected in HTLV-III-infected cells but not in uninfected cells, including normal human tissue,

Received 27 July; accepted 27 September 1984.

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indicating that this virus is exogenous to man. We also demonstrate distant nucleic acid sequence homology between the cloned genome of HTLV-III and those of HTLV-I and HTLV-II. The availability of the cloned HTLV-III genome will now allow an unambiguous comparison of this virus with other retroviruses that also have been associated with the pathogenesis of AIDS⁸⁻¹¹, and moreover, will facilitate the development of diagnostic and therapeutic measures in the treatment of AIDS.

All human retroviruses that have been extensively characterized are lymphotropic, especially OKT4 lymphotropic, and induce formation of multinucleated cells on infection. These viruses also contain a relatively high-molecular weight reverse transcriptase with preference for Mg^{2+} and possess a major core protein of relative molecular mass 23,000–25,000. We named the viruses human T-cell leukaemia viruses, or HTLV, in accordance with recent convention^{12,13}. The first two subgroups of HTLV (I and II) are associated with T-cell malignancies and can transform T cells *in vitro*¹². HTLV-III has many properties in common with HTLV-I and HTLV-II but has cytopathic rather than transforming activity. The crucial step allowing us to isolate and characterize HTLV-III, and to produce sufficient purified viral reagents for serological assays, was the successful transmission of HTLV-III to an immortalized human T-cell line (HT) and to clones derived from this line which were significantly resistant to the cytopathic effects of the virus. This led to the establishment of permanently infected, high-producer cell lines for continuous production of HTLV-III². One of these cell lines, H9/HTLV-III, produces large quantities of HTLV-III and serves as the principal producer cell line for immunological assays used to detect virus-specific antigens and antibodies in sera from AIDS patients. The uninfected parental cell line (HT) and its derivatives (H9 and H4) were negative by all criteria for retrovirus infection, including HTLV-I, HTLV-II and HTLV-III (M.P., in preparation). To clone the HTLV-III genome, we isolated unintegrated viral DNA after acute infection of H9 cells with concentrated HTLV-III and cloned this DNA into a λ phage library to be screened with viral cDNA.

Concentrated virus from the H9/HTLV-III cell line was used to infect fresh uninfected H9 cells at a multiplicity of 50 viral particles per cell and cultures were collected after 4, 10, 15, 24 and 48 h. Extrachromosomal DNA was extracted according to the procedure of Hirt¹⁴ and assayed for its content of unintegrated viral DNA using HTLV-III cDNA as a probe. The synthesis of this cDNA was primed with oligo(dT) and reverse-transcribed from poly(A)-containing RNA of virions that had been banded twice on sucrose density gradients¹⁵. Unintegrated linear viral DNA was first detected after 10 h and was also present at the subsequent time points. Figure 1 shows a Southern blot of the 15-h sampling. A band of ~10 kilobases (kb) in the undigested DNA represents the linear form of unintegrated

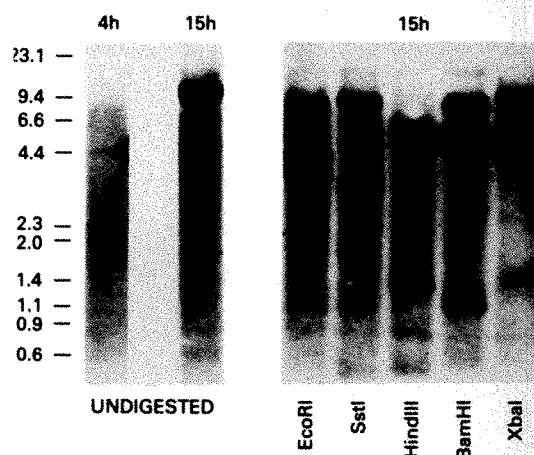


Fig. 1 Southern blot²² analysis of unintegrated HTLV-III DNA. No viral sequences could be detected in the undigested DNA after 4 h. However, a major species of viral DNA ~10 kb long was present at 10, 15, 24 and 48 h, representing the linear unintegrated form of the virus. The figure shows a representative Southern blot of the 15-h sample digested with several restriction enzymes.

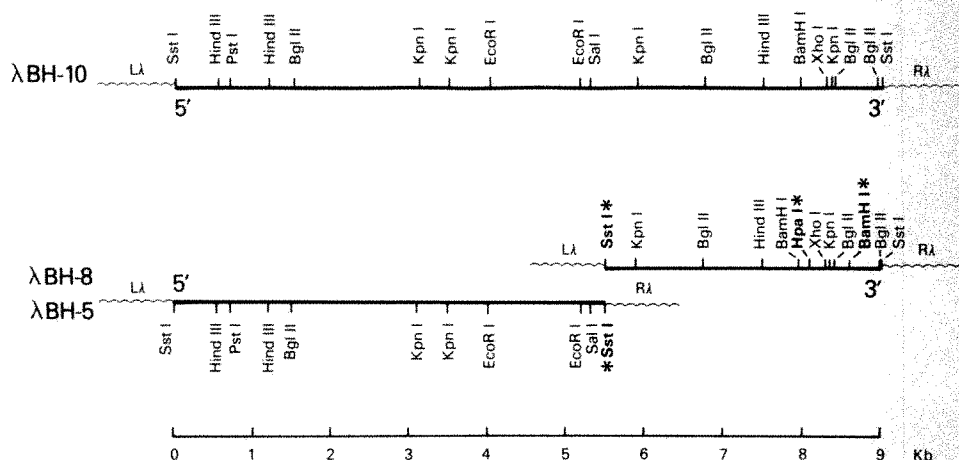
Methods: Fresh uninfected H9 cells (8×10^9) were infected with concentrated supernatant from cell line H9/HTLV-III containing 4×10^{11} particles of HTLV-III. Infected cells were divided into five roller bottles and collected after 4, 10, 15, 24 and 48 h. Low-molecular weight DNA was prepared using the Hirt fractionation procedure¹⁴ and 30 μ g of undigested and digested DNAs were separated on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized to an HTLV-III cDNA probe for 24 h at 37 °C in $2.4 \times$ SSC, 40% formamide and 10% dextran sulphate. cDNA was synthesized from poly(A)-selected RNA prepared from doubly banded HTLV-III virus in the presence of oligo(dT) primers²³. Filters were washed in $1 \times$ SSC at 65 °C.

HTLV-III. No closed or nicked circular DNA could be detected at 10, 15 or 24 h, but both forms were evident in small amounts at 48 h (data not shown). The viral genome was not cleaved by *XbaI*, whereas *SstI* generated three predominant bands of 9, 5.5 and 3.5 kb (Fig. 1). We interpreted these bands as representing the genomes of two forms of HTLV-III, both cut by *SstI* in or near the long terminal repeat (LTR), and one having an additional *SstI* site in the middle of its genome. The other enzymes generated a more complex pattern of restriction fragments requiring cloned DNA for further analysis.

Figure 2 shows the restriction map of three clones, designated λ BH10, λ BH5 and λ BH8, which correspond in size to the three *SstI* fragments shown in Fig. 1. Comparison of these maps suggests that λ BH5 plus λ BH8 constitute one HTLV-III

Fig. 2 Restriction endonuclease map of two closely related HTLV-III forms cloned from unintegrated viral DNA. Three recombinant clones (λ BH10, λ BH5 and λ BH8) were analysed and their inserts (9, 5.5 and 3.5 kb, respectively) were mapped with the indicated enzymes. Together they represent two genomic equivalents of HTLV-III that are highly related but differ in three enzyme sites, indicated by bold letters and asterisks.

Methods: Low-molecular weight DNAs pooled from the 15- and 24-h samples were fractionated on a 10–40% sucrose gradient²³. Aliquots of the fractions were electrophoresed on a 0.5% agarose gel, transferred to nitrocellulose paper and hybridized to HTLV cDNA in conditions described in Fig. 1 legend. Fractions containing the unintegrated linear HTLV-III genome shown by hybridization



were pooled; the DNA was subsequently digested with *SstI*, then ligated to phosphatase-treated *SstI* arms of λ gtWes- λ B. After *in vitro* packaging, recombinant phages were screened for viral sequences with HTLV-III cDNA^{15,23}.

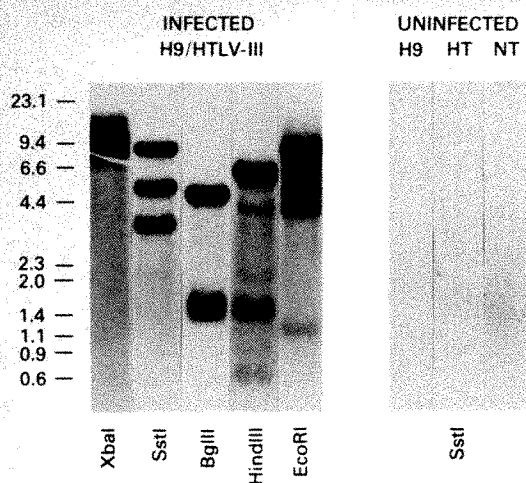


Fig. 3 Demonstration of the presence of HTLV-III viral sequences in the infected cell line, H9/HTLV-III. Both variant forms of HTLV-III defined by differences in *SstI* sites were detected in H9/HTLV-III DNA. No HTLV-III sequences were found in uninfected H9 cells, uninfected HT cells or normal human thymus (NT). **Methods:** High-molecular weight DNA (10 µg) was digested with restriction enzymes as indicated and hybridized to the nick-translated phage insert from λ BH10 in the conditions described in Fig. 1 legend.

genome, and λBH10 another. The two viral forms differ in 3 of 21 mapped enzyme sites, including the internal *SstI* site. As expected, the phage inserts of λBH5 and λBH8 hybridize in high-stringency conditions ($T_m = -25^\circ\text{C}$) to λBH10 but not to each other, as analysed by Southern blot hybridization and electron microscopic heteroduplex analysis (data not shown). To determine the orientation of the three clones, we used as a probe a cDNA clone (C15) containing U3 and R sequences (S.K.A. *et al.*, in preparation); C15 hybridized strongly to the 0.5 kb *BglII* fragment of λBH10 and λBH8, orienting this side 3'. Assuming that *SstI* cuts only once in the vicinity of the HTLV-III LTR, the clones λBH10 and λBH5/λBH8 represent two complete genomic equivalents of the linear unintegrated form of HTLV-III that vary in three restriction enzyme sites. However, the viral fragments cloned into λBH5 and λBH8 may have been derived from the same or two different viruses.

The presence of two variant forms of HTLV-III in the original cell line was demonstrated by hybridizing the radiolabelled insert of λBH10 to a Southern blot of H9/HTLV-III genomic DNA digested with several restriction enzymes (Fig. 3); both forms were detected using *SstI*, which generated the expected three bands of 9, 5.5 and 3.5 kb. *XbaI*, which does not cut the provirus, generated a high-molecular weight smear representing polyclonal integration of the provirus, plus a band of ~10 kb. This 10-kb band was also detected in undigested H9/HTLV-III DNA (not shown), indicating that it represents unintegrated viral DNA. The presence of unintegrated viral DNA also explains the 4- and 4.5-kb *EcoRI* fragments seen in both the Hirt and total cellular DNA preparations (Figs 1, 3). Both *BglII* and *HindIII* cut within the LTR and generate the expected internal bands. Several faint bands in addition to the expected internal bands generated by *HindIII* digestion, represent either defective proviruses or other variant forms of HTLV-III present in low copy number.

The absence of HTLV-III sequences from the DNA of the uninfected H9 cell line, the uninfected parental cell line HT and normal human thymus (Fig. 3), demonstrates clearly the exogenous nature of HTLV-III and shows that the virus does not contain human cellular sequences. The same results were obtained using inserts from λBH5 and λBH8 as probes.

The availability of the cloned HTLV-III genome also allowed us to evaluate sequence homology between HTLV-III and other members of the HTLV family including HTLV-I and HTLV-II,

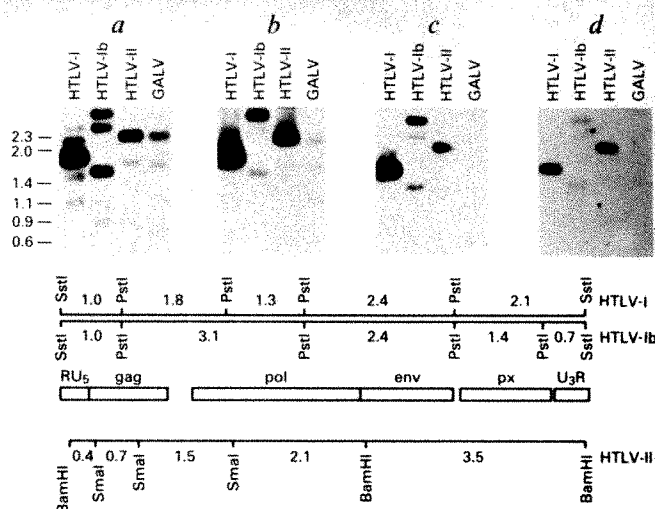


Fig. 4 Sequence homology of HTLV-III to other members of the HTLV family. Schematic restriction maps of HTLV-I, HTLV-Ib and HTLV-II are shown at the bottom, indicating the length (in kb) and location of the generated fragments with respect to the corresponding genomic regions of HTLV-I. LTR, *gag*, *pol*, *env* and *pX* regions are drawn to scale according to the published nucleotide sequence of HTLV-I²⁴. The bands that are most highly conserved as stringency increases correspond to the *gag/pol* junction region of HTLV-I (1.8-kb *PstI* fragment) and HTLV-Ib (3.1-kb *PstI* fragment) and to the 3' part of the *pol* region of HTLV-II (2.1-kb *SmaI/BamHI* fragment) which do not overlap assuming that HTLV-II has a genomic organization similar to that of HTLV-I. Fragments corresponding to *pX* of HTLV-I (2.1-kb *SstI/PstI* fragment) and HTLV-Ib (1.4-kb *PstI* fragment) are only faintly visible at $T_m = -28^\circ\text{C}$ on the original autoradiogram. Digestion of GaLV generates six fragments, none of which hybridizes with HTLV-III in medium or high stringency conditions ($T_m = -42^\circ\text{C}$ and -28°C).

Methods: Subclones of full-length genomes of a prototype HTLV-I (unpublished), HTLV-Ib¹⁶, HTLV-II²⁵ and GaLV (Seato strain)²⁶ were digested with the following enzymes; *PstI* plus *SstI* (HTLV-I and HTLV-Ib); *BamHI* plus *SmaI* (HTLV-II); and *HindIII*, *SmaI* and *XhoI* (GaLV). Four replicate filters were prepared and hybridized for 36 h under low stringency (8×SSC, 20% formamide, 10% dextran sulphate at 37°C) to nick-translated insert of λBH10. Filters were then washed in 1×SSC at different temperatures: a, 22°C ($T_m = -70^\circ\text{C}$); b, 37°C ($T_m = -56^\circ\text{C}$); c, 50°C ($T_m = -42^\circ\text{C}$); and d, 65°C ($T_m = -28^\circ\text{C}$), and subsequently autoradiographed for 24 h.

as well as a variant of HTLV-I (HTLV-Ib) recently isolated and molecularly cloned from a Zairian patient with adult T-cell leukaemia¹⁶. Replicate Southern blots of restriction enzyme-digested clones comprising the complete genomes of HTLV-I, HTLV-Ib and HTLV-II, and of gibbon ape leukaemia virus (GaLV) as a control, were hybridized with the full-length HTLV-III probe (BH10) in relaxed conditions, after which the filters were washed in conditions of low, medium and high stringency (Fig. 4). This experiment demonstrates homology between HTLV-III and HTLV-I, HTLV-Ib and HTLV-II, but not between HTLV-III and GaLV. Hybridization of HTLV-III with other members of the HTLV family could be detected in conditions ($T_m = -42^\circ\text{C}$ and -28°C) where no hybridization to GaLV was seen (Fig. 4c, d). Note that the restriction fragments showing greatest homology to HTLV-III correspond to the *gag/pol* region of HTLV-I and to an apparently non-overlapping portion of the *pol* region of HTLV-II (assuming that the genomic arrangement of HTLV-II is similar to that of HTLV-I). Hybridization to a fragment containing exclusively *pX* sequences in HTLV-Ib (1.4-kb *PstI* fragment) and to the corresponding fragment in HTLV-I containing *pX* and LTR sequences (2.1-kb *PstI/SstI*) was detectable at $T_m = -28^\circ\text{C}$ but was very faint. *pX* sequences of HTLV-II did not hybridize to the HTLV-III probe in the same stringency conditions, nor did fragments containing LTR or envelope sequences of both HTLV-I and HTLV-II.

Overall, these findings using the cloned HTLV-III probe agree with our previous observations using HTLV-III cDNA¹⁵, which also revealed sequence homology, especially in the *gag/pol* regions of the HTLV-I, HTLV-II and HTLV-III genomes. However, we emphasize that HTLV-III is much less closely related to HTLV-I and HTLV-II at the nucleic acid level than HTLV-I and HTLV-II are to each other^{17,18} and that this homology is most evident in the *gag/pol* region of these viruses under stringent hybridization.

Thus, we have molecularly cloned two closely related but distinguishable genomic equivalents of HTLV-III from the H9/HTLV-III cell line, which has been the principal source for all viral reagents used in studies of the sero-epidemiology of HTLV-III in AIDS patients¹⁻⁷. Note that this virus from the H9/HTLV-III cell line retains its cytopathic activity against fresh normal human lymphocytes (unpublished data). Using these clones as probes, we also detected HTLV-III viral sequences in infected cell lines other than H9/HTLV-III that were established from different AIDS patients, and in fresh uncultured lymphoid tissues of AIDS patients¹⁹. These findings suggest that the cloned HTLV-III genomes reported here represent the probable aetiological viral agent of AIDS. The finding of two variant forms of HTLV-III in the H9/HTLV-III cell line could reflect cumulative *in vitro* mutations in a highly replicative virus. The two forms could also represent different isolates as, when first established, the H9/HTLV-III cell line was infected with pooled material from several different AIDS patients². Preliminary studies of other HTLV-III isolates indeed indicate that HTLV-III, unlike HTLV-I and HTLV-II, exhibits substantial diversity in its restriction enzyme cleavage pattern when isolates from different patients are compared¹⁹. Further characterization and sequence analysis will help to define the natural variability of this virus, which has important implications with respect to its pathogenicity and origin, and attempts at preventive measures for AIDS. The availability of the cloned HTLV-III genome should also now allow direct comparison of this virus with a similar group of retroviruses described by other investigators⁸⁻¹¹ which has also been linked to the pathogenesis of AIDS and which appears to be immunologically and morphologically indistinguishable from HTLV-III (M. Sarngadharan *et al.*, unpublished). Finally, the demonstration of a substantial amount of unintegrated viral DNA in the chronically infected cell line H9/HTLV-III, distinguishes HTLV-III from HTLV-I, HTLV-II and most other retroviruses. It will be important to determine whether the presence of unintegrated DNA has a role in the cytopathicity of HTLV-III, as has been proposed for certain other retroviruses^{20,21}.

Received 9 August; accepted 27 September 1984.

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Metabolic oxidation phenotypes as markers for susceptibility to lung cancer

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That bronchial carcinoma is not an inevitable consequence of cigarette smoking has stimulated the search for host factors that might influence the susceptibility of the individual smoker. One plausible host factor would be a polymorphic gene controlling the metabolic oxidative activation of chemical carcinogens, giving rise to wide inter-subject variation in the generation of cancer-inducing and/or promoting species. Recently, three genetic polymorphisms of human metabolic oxidation have been demonstrated (as characterized by debrisoquine, mephenytoin and carbocysteine), with the metabolism of several substrates exhibiting the phenomenon¹⁻³. Debrisoquine 4-hydroxylation segregates into two human phenotypes, each comprising characteristic metabolic capability⁴⁻⁶. We report here the frequency of debrisoquine 4-hydroxylation phenotypes in age-, sex- and smoking history-matched bronchial carcinoma and control patients. Cancer patients showed a preponderance of probable homozygous dominant extensive metabolizers (78.8%) with few recessive poor metabolizers (1.6%) compared with smoking controls (27.8% and 9.0% respectively). We conclude that the gene controlling debrisoquine 4-hydroxylation may be a host genetic determinant of susceptibility to lung cancer in smokers and that it represents a marker to assist in assessing individual risk.

The metabolism of debrisoquine was examined in 479 cigarette smokers who had or had not presented with bronchogenic carcinoma, in order to determine the frequency of extensive metabolizer (EM) and poor metabolizer (PM) phenotypes in each group. Patients were recruited from areas of London within the Islington District, Bloomsbury District and Wandsworth District Health Authorities and were admitted primarily to Chest Unit beds at Whittington Hospital. All were white Europeans with a positive history of cigarette smoking (>20 pack-yr, that is, number of packs of 20 cigarettes per day × number of years of smoking). Subjects were excluded if chemotherapy or drugs known to interfere with the phenotyping test¹ had been given, if there were signs of abnormal hepatic or renal function and if additional acute conditions such as heart failure or severe chest infection obtained. The cancer patients ($n = 245$) had a definite diagnosis of bronchogenic carcinoma proven by histology (108), cytology (85) or histology/cytology (44) from samples obtained at bronchoscopy (194), transcutaneous needle biopsy (24), mediastinoscopy (9) and pleural biopsy (6). Cell types comprised squamous cell (138), small cell (68), large cell (8) and undifferentiated (1) carcinomas, together with 30 adenocarcinoma patients. Control patients ($n = 234$) were smokers with chronic airflow limitation, without evidence of carcinoma. Each patient received no drugs after 21.30 h the day before the test, nor for 2 h after the start of the test at 07.00 h. They were each given a 10 mg debrisoquine tablet orally; all urine was collected for the subsequent 8 h and analysed for its content of debrisoquine (D) and 4-hydroxydebrisoquine (4-HD) by electron-capture gas chromatography⁷. The metabolic ratio (urinary D/4-HD) thus determined was used to assign phenotype (EM, 0.1-12.6; PM, 12.7-100)⁵. Routine clinical chemistry and haematology were performed on a blood sample from each patient within 2 days before or after the test.

Cancer and control patients were similar in age (66.5 ± 7.4 ($\pm$ s.d.) and 67.2 ± 3.3 yr respectively), sex ratio (M/F) (1.82, 1.89) and smoking history (60.3 ± 24.0 , 59.4 ± 21.1 pack-yr). The results showed that the patients also had similar levels of plasma Na^+ (137 ± 6 , 137 ± 4 mM in cancer and control patients, respectively), HCO_3^- (27.0 ± 5.4 , 26.8 ± 4.9 mM), urea (5.0 ± 1.3 , $5.1 \pm$

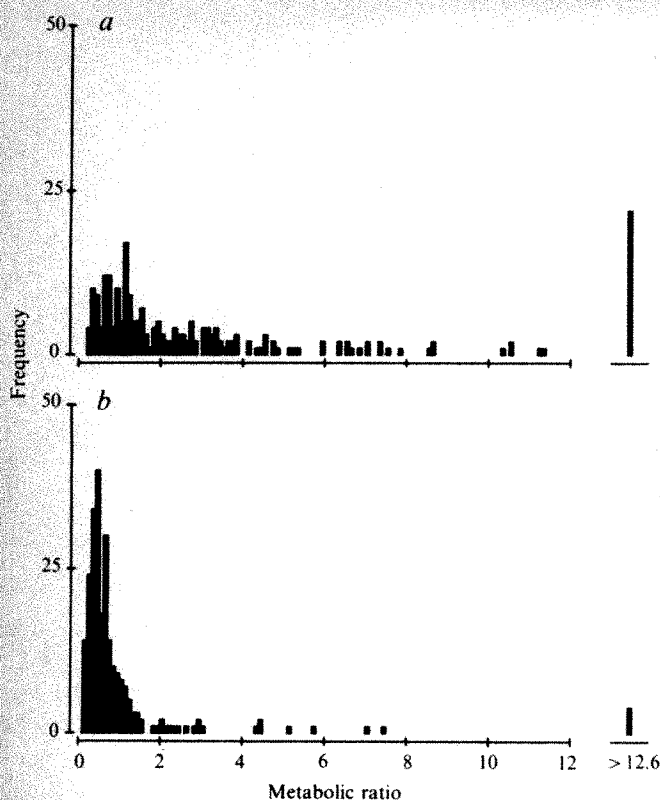


Fig. 1 Distribution of metabolic ratios (urinary D/4-HD) in smoking controls (a, $n = 234$) and bronchogenic carcinoma patients (b, $n = 245$).

1.6 mM), total protein (70.5 ± 4.8 , 70.9 ± 5.0 g l⁻¹ and bilirubin (8.6 ± 8.7 , 8.0 ± 7.6 μ M), and comparable white cell counts (9.0 ± 3.2 , $8.7 \pm 2.5 \times 10^9$ l⁻¹), mean corpuscular haemoglobin concentration (32.7 ± 2.2 , 32.8 ± 2.0 g per 100 ml) and platelet count (321 ± 85 , $325 \pm 89 \times 10^9$ l⁻¹). Values for plasma K⁺, albumin, alkaline phosphatase, aspartate aminotransferase, haemoglobin, packed cell volume, mean corpuscular volume and mean corpuscular haemoglobin all showed small but statistically significant differences between cohorts, but nevertheless fell within the 'normal' clinical range.

Debrisoquine 4-hydroxylation, however, showed several major differences between cancer and control cohorts (Fig. 1). While the metabolic ratios for 'smoking' controls were distributed across the full range of values and included 21 recessives (values > 12.6), the metabolic ratios for cancer patients were aggregated towards the left end (rapid metabolism) of the spectrum, with only four recessives. Figure 2 shows the combined data in logarithmic form, which both compresses and normalizes the distribution. Analysis of family pedigrees and a similar distribution for a healthy white British population⁶ has allowed estimation of the mean ($\pm$ s.d.) log₁₀ metabolic ratios for homozygous EM, heterozygous EM and recessive PM genotypes as -0.26 ± 0.23 , 0.13 ± 0.33 and 1.44 ± 0.31 , respectively. Remarkably, the additive cancer and control distributions have major modes at -0.35 , 0.05 and 1.75 , which almost certainly correspond to the three genotypes (Fig. 2). Thus, most cancer patients with log₁₀ metabolic ratios of less than 0 (193/245; 78.8%) probably have homozygous dominant genotypes, whereas the majority of smoking controls (148/234; 63.2%) with log₁₀ values between 0 and 1.1 (Fig. 2; ratios 1–12.6 in Fig. 1) are probably heterozygotes. Probable homozygous individuals in the control group were 27.8% (65/234) EM and 9.0% (21/234) PM genotypes. Finally, inspection of the extremities of the distribution reveals not only that there are no controls with ratios of 0.1–0.2, but also that the PM mode is almost devoid of cancer patients. Of the four cancer patients with a PM phenotype, two were adenocarcinoma patients and one had a 150 pack-yr cigarette consumption.

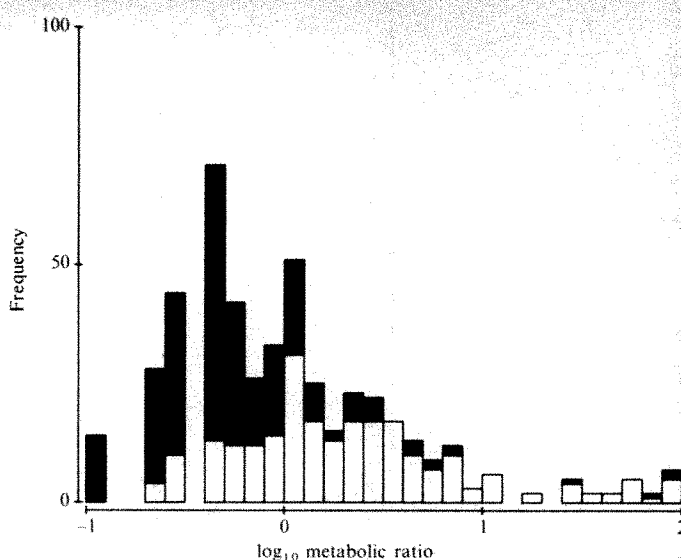


Fig. 2 Distribution of log₁₀ metabolic ratios for cancer (solid bars) and control (open bars) groups combined.

Care has been taken to ensure similarity between the two cohorts for factors which could influence either the disease (age, sex, smoking history) or the phenotyping test (hepatic/renal impairment, ethnicity, recent drug history). Nevertheless, the question arises as to whether or not the metabolic differences between cohorts which we report here contribute to the cause of lung cancer or are a result of it. Several reasons support the former interpretation of these data. First, our own findings in patients with cancer at other sites such as colon, rectum and urinary bladder, suggest that the presence of a tumour has no effect on the phenotyping test. Also, the debrisoquine metabolic ratio arises from hepatic metabolism of the administered drug and therefore major pathological changes within the liver might be expected to perturb the phenotyping test more than any other disease state. Appropriate data from a population of alcoholics with liver diseases of varying severity show that this is not the case⁸.

We propose, therefore, that the metabolic and genetic differences between white European cigarette smokers in this study who developed lung cancer by, on average, their mid-sixties and those who did not, reflect the aetiology of the disease. Homozygous dominants appear to be at high risk of developing cancer, whereas heterozygotes and recessives are at relatively low risk.

Debrisoquine 4-hydroxylase may not be involved in chemical carcinogen metabolism, but its gene may flank others with oncogenic potential, recessives for which will almost certainly be recessives for debrisoquine 4-hydroxylation. Such genes might be structural or regulatory genes for cytochromes P450, such as the *Ah* locus⁹, or indeed genes not involved in chemical carcinogen metabolism, for example, cellular oncogenes. We conclude that the metabolic oxidation phenotypes serve as genetic markers for differential lung cancer susceptibility.

This work was supported by grants from the Cancer Research Campaign. J.R.I. is a Wellcome Trust Senior Lecturer. We thank Nick Oates for computer analysis of the data. We also thank the consultants at St James Hospital, Balham and The National Temperance Hospital who allowed us to study their patients.

Received 11 April; accepted 26 July 1984.

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Alu sequences are processed 7SL RNA genes

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7SL RNA is an abundant cytoplasmic RNA which functions in protein secretion as a component of the signal recognition particle¹. *Alu* sequences are the most abundant family of human and rodent middle repetitive DNA sequences (reviewed in ref. 2). The primary structure of human 7SL RNA consists of an *Alu* sequence interrupted by a 155-base pair (bp) sequence that is unique to 7SL RNA³. In order to obtain information about the evolution of the *Alu* domain of 7SL RNA, we have determined the nucleotide sequence of a cDNA copy of *Xenopus laevis* 7SL RNA and of the 7SL RNA gene of *Drosophila melanogaster*. We find that the *Xenopus* sequence is 87% homologous with its human counterpart and the *Drosophila* 7SL RNA is 64% homologous to both the human and amphibian molecules. Despite the evolutionary distance between the species, significant blocks of homology to both the *Alu* and 7SL-specific portions of mammalian 7SL RNA can be found in the insect sequence. These results clearly demonstrate that the *Alu* sequence in 7SL RNA appeared in evolution before the mammalian radiation. We suggest that mammalian *Alu* sequences were derived from 7SL RNA (or DNA) by a deletion of the central 7SL-specific sequence, and are therefore processed 7SL RNA genes.

The signal recognition particle (SRP) mediates the translocation of secretory proteins across the endoplasmic reticulum⁴⁻⁶. SRP consists of six different polypeptides and one molecule of 7SL RNA¹. Previous studies have shown that 7SL RNA is an essential component for SRP function¹.

In the human genome, *Alu* DNA constitutes the dominant family of short middle-repetitive mobile sequences⁷, and in addition, *Alu* equivalents have been described in the genomes of other primates^{8,9}, as well as in rodents^{10,11}. Human 7SL RNA is an abundant cytoplasmic RNA of 300 nucleotides which was shown to be partially homologous with *Alu* DNA¹². Approximately 100 nucleotides at the 5' end and 45 nucleotides at the 3' end of the RNA are homologous with the human *Alu* right monomer consensus sequence³. The central portion of 155 nucleotides shows no homology with *Alu* DNA and is unique to 7SL RNA (Fig. 1). The entire human and rodent *Alu* sequences are represented in the 7SL molecule (see Fig. 1).

Fig. 2 The nucleotide sequences of *Xenopus* and *Drosophila* 7SL DNA and their comparison with the human 7SL cDNA. The nucleotide sequence of a cDNA copy of *X. laevis* 7SL RNA (top line) and of the *D. melanogaster* 7SL RNA gene (bottom line) were determined by the chemical degradation²⁵ and the dideoxy-chain termination methods²⁶. The anticoding strand of both 7SL DNAs was aligned with the sequence of human cDNA clone 7L1 (ref. 3). The sequences are numbered with respect to the first nucleotide of the human 7SL cDNA. The first four nucleotides of cDNA clone 7L1³ are not included, because they are not represented in human 7SL RNA (E.U., unpublished observation). Asterisks denote homology between human 7L1 DNA and the amphibian or insect molecule, respectively. Dashes are occasionally introduced to maximize homology. The *Alu*-like portions of the human 7SL DNA are boxed.

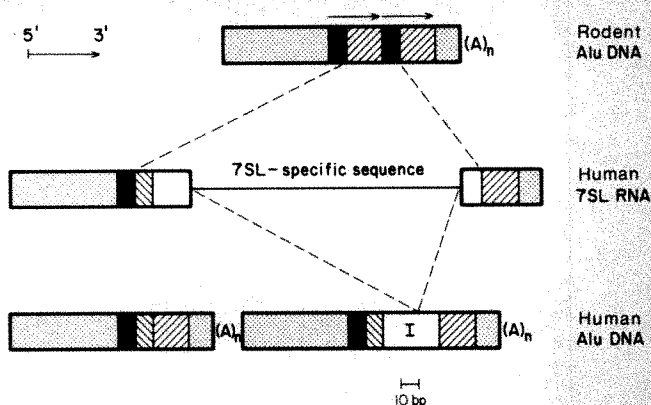
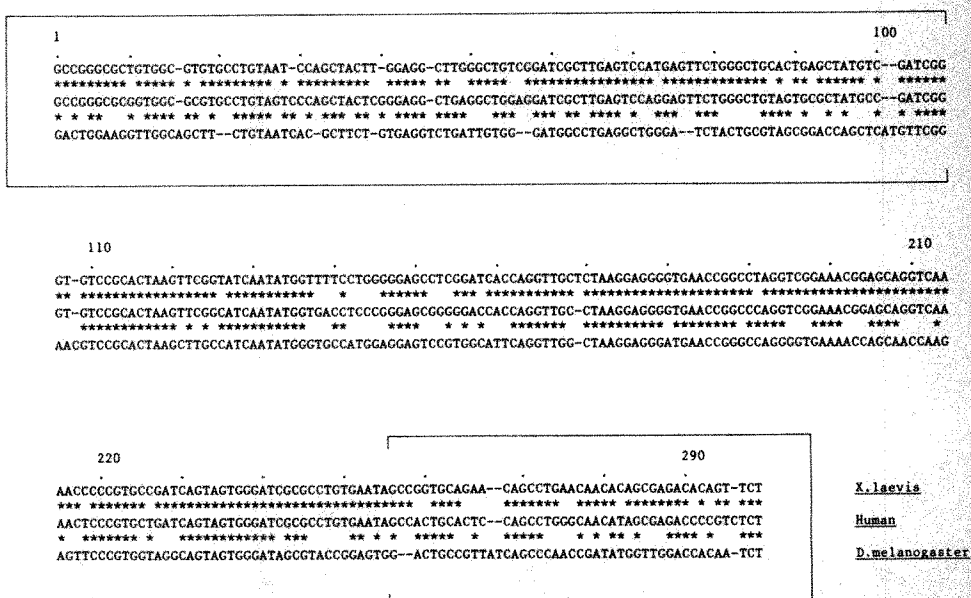


Fig. 1 The structural relationship of human 7SL RNA to the consensus sequences of human and rodent *Alu* DNA. Homologous sequences are indicated by identical shading. Human *Alu* DNA is a head to tail dimer of two similar sequences ~130 bp long. The right monomer contains an insert (I) which is not present in the left half²³. The rodent *Alu* equivalent sequence is a monomer^{10,11}. We have found that the mouse B1 *Alu*-equivalent consensus sequence compiled by Kalb *et al.*²⁴ contains an internal tandem duplication of 30 bp between positions 62 and 121. The type I Chinese hamster *Alu*-equivalent sequence¹¹ has a similar structural arrangement. Arrows above the rodent *Alu* DNA indicate the position of the 30-bp tandem duplication; (A)_n denotes an A-rich sequence which follows the *Alu* sequence at the 3' end.

To obtain information about the evolution of 7SL RNA and its possible relationship to *Alu* sequences, we have analysed the 7SL sequence in *X. laevis* and *D. melanogaster*. A cDNA copy of *in vitro* polyadenylated 7SL RNA of *X. laevis* was prepared as described previously³. For *Drosophila*, a bacteriophage library of embryonic DNA was screened for sequences homologous to the 155-nucleotide portion of human 7SL RNA not homologous with *Alu* DNA, using the 125-base pair (bp) *Sau*3A fragment of clone p7L1 as a probe³. Restriction endonuclease mapping of three independent positive phage clones and subsequent sequence determination of appropriate DNA fragments established that there are two identical genes coding for 7SL RNA in *D. melanogaster* (C.T. and Jean Burckhardt, manuscript in preparation). Figure 2 compares the primary structures of the *Xenopus* and *Drosophila* 7SL DNA with the human counterpart. The length of the 7SL RNAs of *Xenopus* and *Drosophila* is comparable with that of human 7SL RNA, which agrees with the observation that the three 7SL RNAs have the same electrophoretic mobility¹³. However, we have not determined the

precise 5' and 3' ends of *Xenopus* and *Drosophila* 7SL RNA. The *Xenopus* sequence is strikingly homologous to the human one over its entire length. There is only a 13% divergence and the mismatches are distributed along the entire length of the molecule. As these two 7SL DNAs are extremely similar, the appearance of the *Alu*-like sequence in the 7SL RNA molecule evidently preceded the mammalian radiation in evolution.

When the sequence of the *Drosophila* 7SL DNA is compared with its human and amphibian counterparts, there is an ~64% homology; however, extended regions in the central 7SL-specific portion can be seen, where the sequences have been almost perfectly conserved. These 7SL-specific homologies may reflect a strong functional constraint acting on these sequences. The *Alu*-like portions of the human and insect 7SL DNA can be only poorly aligned. Nonetheless, the vestiges of the mammalian *Alu*-like sequence can be recognized in the *Drosophila* 7SL DNA. These results, taken together, strongly suggest that the evolution of the *Alu*-like portions of 7SL RNA has taken place gradually. No dramatic event such as the insertion of the 155-bp 7SL-specific DNA into a human *Alu* sequence, as originally suggested³, can account for the identical composite structure of 7SL RNA in two lower organisms. If 7SL RNA genes were in fact assembled from two different types of sequences, such an event must have occurred before the appearance of insects.

Because the structure of 7SL RNA has been extensively conserved in evolution, we propose that 7SL RNA genes represent the ancestor of the *Alu* sequence and that *Alu* DNA arose from the 7SL RNA information through a deletion of the central 7SL-specific sequence. Such a deletion could have occurred either at the DNA level (through non-homologous recombination within or between 7SL RNA genes) or at the RNA level (due perhaps to an aberrant RNA joining or splicing event).

The discovery of 'processed genes' which lack intervening sequences and closely resemble the mature mRNA structure¹⁴, and the finding of pseudogenes for the small nuclear RNAs¹⁵ have provided evidence that RNA information can flow back into the genome. We speculate that the prototypical *Alu* sequence is a processed 7SL RNA gene. It has been observed that the 7SL RNA molecule within SRP is very resistant to micrococcal nuclease digestion^{1,16}. Interestingly, only three major discrete RNA fragments are generated by limited nuclease digestion of SRP. Two fragments of 72 and 45 nucleotides span the 5' and 3' *Alu*-like portions, respectively, while the third product of nuclease cleavage spans the entire 7SL-specific sequence of the RNA¹⁶. These experiments clearly indicate that an enzyme probe can readily recognize the boundaries of the *Alu*-like and 7SL-specific domains of 7SL RNA in the intact ribonucleoprotein particle. This finding suggests that SRP itself may contain the RNA in a conformation which promotes excision of the 7SL-specific domain. Once this happens, the 5' and 3' *Alu*-like portions could be ligated together, generating a contiguous *Alu* RNA. The sequence of the spliced RNA could then have been re-integrated into the genome.

Several groups have proposed that processed genes could arise from the reverse transcription of cellular RNA species, followed by integration of the cDNA into new chromosomal sites in germ-line DNA^{14,17-20}. In this context, 7SL RNA was first discovered as a component of avian and murine retroviral particles²¹. Because retroviruses encode a reverse transcriptase and carry the enzyme within the virion, it is tempting to speculate that retroviruses are responsible for the generation of an *Alu* sequence from 7SL RNA.

Using the homologous 7SL DNAs as probes, we have searched in the genomes of *Drosophila* and *Xenopus* for 7SL RNA-related sequences (data not shown). We note that the *Drosophila* genome contains only two 7SL RNA genes and no other cross-hybridizing sequences. In contrast, *Xenopus* DNA is rich in sequences capable of cross-hybridizing to the homologous 7SL DNA probe. Some of these sequences might represent *Alu*-like sequences, as suggested by previous studies²². It is possible that the excision of the *Alu* sequence from 7SL RNA (or DNA) may have occurred several times in evolution. Once freed of 7SL

sequences, *Alu* DNA may no longer be subject to the functional constraints which operate on the 7SL RNA genes and thus may be capable of evolving independently from 7SL RNA.

We thank Jean Burckhardt for making the *Drosophila* 7SL recombinant clones available to us, Alan Weiner and Frank Richards for advice and support, Peter Walter for his continuous interest, Alex Young for technical assistance and Manny Ares, Joan Steitz, Peter Walter and Alan Weiner for critical reading of the manuscript. E.U. was the recipient of a long-term EMBO fellowship and is on leave of absence from the Istituto di Biologia Generale, Università di Roma. This work was supported in part by NIH grants GM 31073 and GM 31335 to Alan Weiner and AI 08614 to Frank Richards.

Received 9 July; accepted 8 August 1984.

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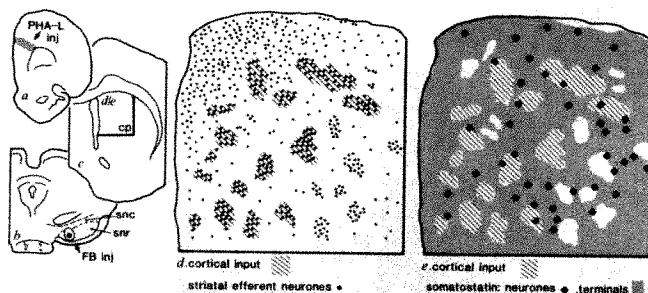
Erratum

The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems

C. R. Gerfen

Nature **311**, 461-464 (1984)

THE key was omitted from Fig. 1. A correct version appears below:



History peopled

W.F. Bynum

The Discoverers: A History of Man's Search to Know His World and Himself.

By Daniel J. Boorstin.

Dent/Random House: 1984. Pp. 768. £15, \$25.

DANIEL Boorstin has been Librarian of Congress since 1975 and his previous dozen books (including one Pulitzer Prize winner) have been on American history. *The Discoverers* is his homage to the rest of the world. America and Americans are of course part of his story, but only a relatively small one. His primary focus is Europe and Asia and those who have contributed to "man's search to know his world and himself".

The book's sub-title and size suggest that Mr Boorstin has set himself a task on the grand scale. A look at the volume's four main sections confirms this: "Time"; "The Earth and the Seas"; "Nature"; "Society". This organization provides a rough chronological thrust to his narrative. "Time" deals with ancient calendars, seasonal reckoning and time-telling from antiquity to the coming of clocks and watches. John Harrison's chronometer for calculating longitude links that section with the next, which examines cosmology and cartography, navigation and exploration, from long before Ptolemy to long after Columbus. The section on Nature takes off from the Scientific Revolution, when the telescope and microscope extended the range of vision, and makes its leisurely way through some of the principal scientific disciplines of the eighteenth and nineteenth centuries, such as taxonomy, palaeontology and evolutionary biology. Finally, "Society" describes a fascinating mélange of topics, from printing and books to the writing of history, the discovery of Troy and Keynesian economics.

The result is both less and more than a conventional history of science. It is less because the coverage is sometimes patchy, particularly of the physical sciences after Newton. There is virtually nothing on chemistry (Lavoisier rates only one passing mention) and two centuries of physics are covered in a rather unfortunate closing chapter (part of "Society", not "Nature") in which Mr Boorstin leaves himself only a single page to dispense with James Clerk Maxwell, J.J. Thomson, Ernest Rutherford and Albert Einstein. Seventeenth-century microscopists such as Leeuwenhoek and Malpighi get full treatment, but there is nothing on cell theory of the nineteenth century. Galen, Vesalius, Paracelsus and Harvey (mistakenly knighted on p. 459) get their due, but virtually no doctors after them except for Freud.

Mr Boorstin has therefore not given his

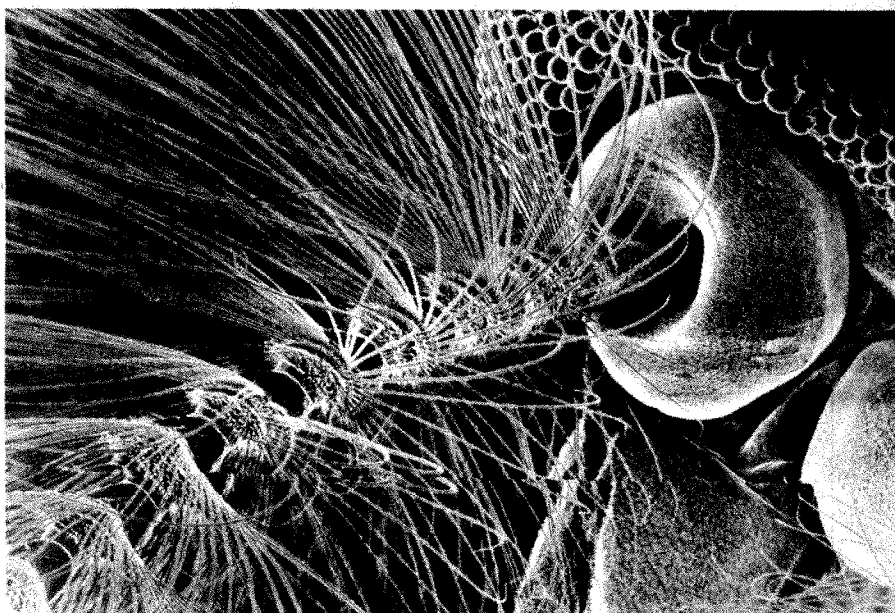
readers much that a single-volume survey of the history of science and scientific discovery should; but he has more than compensated for this lacuna by offering a great deal more. Scattered through this magnificent volume are unexpected themes and people treated with what can only be described as affectionate authority. The Jesuits and their clocks in China; Islamic attitudes to the printing press; the growth of public libraries; the discovery of "prehistory": these and dozens of other vignettes share centre stage with a multinational cast of unfamiliar characters, such as Sir Anthony Panizzi, Italian-born librarian of the British Museum; Martin Waldseemüller, the German clergyman who named America; Chêng Ho, the eunuch Chinese navigator; and Lorenzo Valla, the Italian theological scholar. If the book contains many curious and delightful byways, seekers of more familiar discoveries and discoverers will not be disappointed, for individuals such as Copernicus and Galileo, Henry the Navigator and James Cook, Linnaeus and Darwin, are sympathetically covered. The level of accuracy throughout is unusually high in a book which covers such a vast terrain. A 30-page bibliographical essay guides the reader to the range of sources from which material has been drawn.

Mr Boorstin seems particularly to enjoy biography, and most of the book's 82

chapters are arranged around the achievements of two or three relevant people. This penchant for biography can be seen by the frequency with which he uses biographical metaphors and similes: Karl Marx was "a latter-day Paracelsus", Paracelsus in turn was "a medical Don Quixote". Newton was "the Galahad of the Scientific Quest", whereas several individuals (John Dalton, for example) were Columbuses. Boorstin has a fine eye for anecdote, is particularly good on word derivations (among them "punctual", "travel", "hour", "Bible", "culture") and writes always with elegance and zest. The volume is studded with memorable phrases and epigrams. One such epigram, not directly from his pen but quoted from Callimachus (305–240 BC), head librarian in Ptolemaic Alexandria, declares that "A big book is a big nuisance". If this is true, *The Discoverers* is the exception that proves the rule, for, like the Bible, this is a Good Book. It is best read slowly and in small doses, in order to savour the richness of the story. It is also best read with an historical atlas nearby, for despite the importance of geographical exploration among Boorstin's themes, there are no maps provided. Astronomical diagrams would also have helped readers grasp the differences between the cosmologies of the Babylonians, the Indians, Ptolemy and Kepler.

This book had its origins, Mr Boorstin tells us, in his first visit to Florence a half-century ago, and has been 15 years in the specific researching and writing. This long gestation has produced a splendid brain-child, a wise and optimistic volume which enlightens and entertains. Read it. □

W.F. Bynum is at the Wellcome Institute for the History of Medicine, London.



Seeing sense — base of the antenna of a male mosquito, photographed by Lennart Nilsson. The picture is reproduced from *Nature Magnified*, a collection of Nilsson's work published by Macdonald price £9.95.

Sprung from chains

Struther Arnott

Principles of Nucleic Acid Structure.

By Wolfram Saenger.

Springer-Verlag: 1984. Pp. 556. Pbk DM 79, \$29.50.

FROM time to time one reads a book or an account of an experiment and wishes, with more pleasure than envy, that one had produced it oneself. Wolfram Saenger's book is such a virtuoso production: well-written, profusely illustrated and astonishingly comprehensive. In it, fact and hypothesis are distinguished with rare scrupulousness. Any serious student or researcher will find it a mine of information. The merely curious now have a guidebook with which safely to explore the labyrinth of DNA and RNA structural studies.

Particularly praiseworthy is the way Saenger has managed to deal thoroughly but interestingly with all the fundamental business of conformational nomenclature and crystallographic esoterica. He has not failed to explore the many structural hypotheses still twisting slowly in the wind, but almost nowhere does he confuse these with experimentally well-founded models.

About a third of the book is devoted to exploring the nature and provenance of the polymorphism of DNA and RNA. Any polymer constructed with residues as complex as nucleotides should be extremely polymorphic. Even when restrained in plectonemically wound duplexes, with bases paired and stacked, polynucleotides have many degrees of structural freedom. Yet polymorphism in DNA and RNA structures has been persistently ignored or belittled, as a brief glance at history will show.

Astbury's early fibres were mixtures of A-DNA and B-DNA, but he interpreted their X-ray patterns in terms of a single allomorph with the residue repeat of B-DNA (0.34 nm) and the structural repeat of A-DNA (2.80 nm). Then, during the renaissance of X-ray studies in the early 1950s, Franklin and Gosling established that DNA was at least dimorphic. Provocatively, they named their newer form A, and the form worked on earlier by Wilkins, B. During the long process of reconciling the Watson-Crick hypothesis with a tangible structure, attention was focused on the more hydrated B form, and A-DNA was forgotten until the 1960s when RNA duplexes were discovered to have A-like structures. Despite conformational similarities the A structures are quite polymorphic, with helix pitches ranging from 2.8 nm to 3.9 nm and major grooves of strikingly variable widths. In the eponymous A-DNA this groove is clenched shut, excluding even water molecules, while in the A duplexes of largest pitch the

major groove is wide enough to accommodate a third polynucleotide chain. B-DNAs have been shown to be just as polymorphic with pitches ranging from 2.4 nm to 3.6 nm.

With Polonius-like retrospective wisdom one would expect the apotheosis of A and B polymorphism to be heteronomous duplexes in which one chain carries C3'-endo-puckered deoxyribose rings like the A structures and the other carries C2'-endo-puckered rings like B. The synthetic duplex poly d(A).poly d(T) indeed has such a structure. (Sadly this particular allomorph arrived too late to catch the Saenger omnibus.) Heteronomous duplexes, unlike allomorphs with identical chains, are highly polar. Perhaps bidirectional sequences on DNA in chromosomes function as valves or direction markers.

Clearly the great variety of (right-handed) helical structures trapped in fibres before 1980 implies at least as great a variability in DNAs and RNAs in other milieux. Saenger's book certainly exposes us to variety in the catalogue of static structures, but the lively world of nucleic acids, rich in movement, bending, breathing, vibrating and shimmering is largely unexplored. Perhaps the *frisson* of the discovery of left-handed DNA will emphasize the reality of polynucleotide variety and variability — but perhaps not!

The possibility of left-handed polynucleotide duplexes was recognized early on: Maurice Wilkins explored sinister

models of A- and B-DNA in the 1950s; later, others touted left-handedness for the D form of poly d(IC).poly d(IC), in hope, but erroneously. When a left-handed (Z) allomorph was eventually observed, ironically it indeed needed alternating purine-pyrimidine sequences. Z-like conformations have been observed with d(GC)_n.d(GC)_n, d(AC)_n.d(GT)_n and d(As⁴T)_n.d(As⁴T)_n but not yet with d(IC)_n.d(IC)_n — nor with d(AT)_n.d(AT)_n.

Depressingly, DNA is again being discussed in dimorphic terms; however now it is B and Z, not A and B! But Z-DNA is polymorphic too: relatively underwound duplexes with the peculiar dinucleotide motif of Z-DNA exist with residue rotations of 0°, -25°, -51° as well as the -60° of the first left-handed structure.

Current X-ray structural studies focus on oligonucleotides in single crystals and in poly(oligonucleotides) in fibres. These structures are less regular. Whether or not the excursions from regularity are related to base sequences is a lively issue. More ambitiously — and perhaps more wisely — there are increasingly successful studies of DNA bound to histones and other proteins which will reveal specifically how variety and variability in the genetic material can be exploited biologically. Among all its other riches, Saenger's book makes us aware of these too. □

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Magnetic mixture

D. Walton

Rock and Mineral Magnetism.

By W. O'Reilly.

Blackie/Chapman & Hall: 1984. Pp.220. £19.50, \$45.

A number of texts on rock magnetism have appeared over the past 20 years, but there is still a clear need for a solid, carefully written book on the subject. Sadly, this contribution does not fit the bill.

In general, the objective of books of this nature is to provide geophysicists with the information to be able to understand how the magnetic vector is produced in their samples. To do this it is essential first to understand the mineralogy, that is, the chemical and physical properties of the magnetic minerals and how these can change with temperature, pressure and chemical environment. Second, it is necessary to understand the physics — how the magnetic moments of individual grains arise, and how assemblies of such grains produce the resultant magnetic vector of the sample given the conditions of temperature, magnetic field, cooling rate and so on to which the sample was exposed.

Dr O'Reilly certainly meets the first of

these goals. There is a wealth of useful information on the mineralogy of magnetic minerals; and while it is quite difficult to find the desired data, the fairly complete index mitigates the problem to a great extent.

Just as certainly, however, the book falls short of the second requirement: the mathematics is quite inadequate, with formulae simply being presented with no derivation and often supported by no reference. The equations are not numbered, often they are not displayed, simply appearing in the body of the text. Furthermore, the author is not only selective in his choice of references (perhaps understandably), but much of the literature he has opted to quote is oversimplified to such an extent that it is wrong. For example only one of Neel's famous papers is referred to, although these contain the basic physics and have not been superseded.

Neel's papers still form the basis for all rock magnetic theory. In discussing the physics of the subject, authors of textbooks would be well advised to begin with them. And if they intend to provide an accurate and reliable source of information for the uninitiated, it might be safer to end with them. □

D. Walton is Professor of Physics at McMaster University, Hamilton, Ontario.

Making a meal of primate ecology

R.D. Martin

Adaptations for Foraging in Nonhuman Primates: Contributions to an Organismal Biology of Prosimians, Monkeys, and Apes.

Edited by Peter S. Rodman and John G.H. Cant.

Columbia University Press: 1984. Pp. 351. Hbk \$45.50; pbk \$18.50.

FOR sound ecological reasons, field-studies of non-human primates have increasingly focused on quantitative studies of feeding behaviour (taken in the widest sense). Food provides the energy required for all activities, but collection incurs a cost, particularly in terms of locomotor energy expenditure, and the overall cost-benefit equation is clearly of central importance in our understanding of the behavioural ecology of any species. That equation, however, contains a large number of different variables and thus a wide-ranging approach to it is necessary.

Adaptations for Foraging in Nonhuman Primates, a collection of 11 chapters edited by Peter Rodman and John Cant, brings together accounts of a variety of different aspects of primate feeding adaptations with this end in mind. The book is, in fact, the outcome of a special symposium on primate "foraging" held at the annual meeting of the American Association of Physical Anthropologists in 1980.

Rodman and Cant have taken an extremely broad view of primate feeding adaptations, including a great number of morphological, physiological and behavioural components. Clearly, an integrated approach to all of these components is much to be desired. This is certainly true with respect to evolutionary biology, and it is gradually being recognized that it is also likely to apply to behavioural ecology.

To take just one example, abundantly illustrated in the book: Kleiber's Law governing the scaling of basal metabolic rate has far-reaching implications for morphology, physiology and behaviour, because of its direct relevance to energy budgets. Complex feedback relationships are involved and explanatory models are really inadequate without some appreciation of them. In leaf-eating primates, for instance, the overall energy budget depends not only on behavioural factors, but also on special adaptations in morphology (such as enlargement of part of the digestive tract to house a flourishing gut flora) and in physiology (such as possession of special physiological capacities for coping with plant secondary compounds). Indeed, it now seems likely that most or all leaf-eating primates exhibit relatively low basal metabolic rates, after body size has been taken into account, and this con-

stitutes part of their overall "strategy". Some of this interdependency is neatly illustrated in Milton's thoughtful chapter on the role of food-processing factors in primate food choice.

There is, of course, a problem involved in taking such a wide-ranging view of the subject. It is all very well being presented with series of tasty dishes; at the end of the meal, digestion must be feasible. In this respect, comparisons are bound to be made with *Primate Ecology*, edited by Tim Clutton-Brock and published by Academic Press in 1977, which very effectively brought together the results of a score of good, quantitative field studies of primate feeding behaviour, but which devoted little attention to morphology, physiology or locomotor behaviour.

Some of the chapters in the present volume are gems in their own right, either as useful syntheses or as accounts of new developments. Among the former, Kay's chapter on morphological correlates of feeding behaviour is especially noteworthy in bringing together several different lines of (predominantly dental) evidence that permit quite reliable inferences to be made about broad dietary habits from anatomical features. This has evident benefits for the interpretation of primate fossils, but the link with energy budgets of living primates is somewhat tenuous.

Of the contributions dealing with novel approaches, Milton's chapter centres on a broad, cross-species comparison of transit times for food material passing through the gut. This adds a new dimension to studies of gut parameters in relation to primate dietary habits. Two further chapters examine locomotion in specific relation to feeding behaviour; Crompton reports on a study of two bushbaby species in South Africa, while Garber describes a study of Geoffroy's tamarin in Panama. Both accounts are notable for their admirable emphasis on quantitative data and for their careful extraction of points for an explanatory framework. Although it is argued in other chapters that there is no clear relationship between diet and locomotor patterns across species, these two studies definitely demonstrate that locomotion is intimately related to food-gathering within species.

A number of authors here use comparisons of two species in order to investigate underlying relationships. The most suc-

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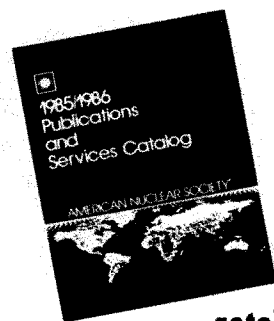


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cessful of them is Waser, who compares two mangabey species. His chapter is actually most remarkable for its careful documentation of the close similarity in behavioural ecology between the arboreal mangabey species (*Cercocebus albigena*) that he studied in Uganda and the largely terrestrial species (*C. galeritus*) studied by Katherine Homewood in Kenya. Waser attempts to relate the relatively limited differences between the two species to ecological differences, but it is somewhat difficult to test the inferences made. This problem is even more acute with other chapters based on a comparison between two species. For instance, Temerin, Wheatley and Rodman attempt to analyse the relationship between body size and foraging in primates by comparing *Macaca fascicularis* (the crab-eating macaque) with *Pongo pygmaeus* (the orang-utan). These two primates do, of course, differ in numerous ways, but a comparison between them cannot reliably reveal which of those differences is due to body size alone. (Curiously, the book includes no allometric analyses other than those contained in Kay's chapter; this technique has much to offer, especially in identifying body-size effects.)

Finally, special mention should be made of Post's essentially theoretical, but

nonetheless challenging chapter arguing that current optimal foraging models are generally designed for fine-grained habitat conditions and are therefore inadequate for interpretation of primate feeding behaviour in patchy habitats.

Perhaps the most surprising point about the book as a whole is that there is no explicit discussion of the crucial distinction between "foraging" (the search for food) and "feeding" (actual ingestion). These terms are very loosely used in the literature and a clearing-up exercise is long overdue. But the book's principal drawback resides in its lack of cohesiveness; in the event, the meal is pretty indigestible. The introduction by Rodman and Cant is interesting in a historical sense, but theoretically superficial, while the closing chapter by Cant and Temerin, which could have served to bring the key points together, actually dissolves into a diffuse listing of large numbers of potential interacting variables. So although *Adaptations for Foraging in Nonhuman Primates* contains much of value in terms of individual chapters, it falls far short of the synthetic view of primate feeding adaptations that should one day emerge. □

R.D. Martin is Professor of Physical Anthropology at University College London.

Piecing it together

John M. Charap

The Ideas of Particle Physics: An Introduction for Scientists.

By J.E. Dodd.

Cambridge University Press: 1984. Pp. 202. Hbk £22.50, \$44.50; pbk £8.95, \$18.

THE IDEAS of particle physics are probably no more difficult than those of many other sciences, but they are certainly less familiar. Notwithstanding the fact that the discoveries of recent years together represent one of the great achievements of modern science, most non-physicists would be hard pressed to explain why there is such confidence in the validity of the "standard model" of electroweak and QCD interaction between leptons and quarks, still less to point beyond present frontiers to more or less credible speculation.

There has long been a need for a book which would enable scientists from other disciplines to share more fully in the continuing endeavour of particle physicists, pursued on a grand scale and addressing the most fundamental question about the ultimate structure of matter; after all, the advances of the past few years have brought about as profound a revolution of our knowledge of the basic constituents of all matter and of their interactions as did the heroic work of Bohr and Rutherford for our understanding of the constitution of atoms. Dr Dodd has written just such a book.

The stage is set with a brief introduction to the key ideas of relativistic quantum field theory, and the drama is allowed to unfold in a broadly chronological sweep. The mathematical demands are modest. Equations are used to summarize or to illustrate rather than to advance arguments. Diagrams likewise are there to elucidate and not to baffle, and the ubiquitous Feynman diagrams need hold no terror. The fact that there are rules which relate them quantitatively to measurable observables is mentioned but no such calculations are undertaken. And the book is bang up to date; the evidence for the top quark from the CERN $p\bar{p}$ collider is the only major post-publication discovery omitted.

If you consider yourself to be reasonably numerate, and want to learn about quarks and gluons, asymptotic freedom and colour confinement, electroweak unification, and W and Z bosons, Dr Dodd's book is an excellent place to start. More important, if you want to know how the jig-saw puzzle seems to be falling into place, making sense of the plethora of particles, then this is the book to read. □

John M. Charap is Professor of Theoretical Physics and Dean of the Faculty of Science at Queen Mary College, University of London.

Hows (and whys) in molecular biology

Peter Little

Transcription and Translation: A Practical Approach.

Edited by B.D. Hames and S.J. Higgins. IRL Press: 1984. Pp. 328. Pbk £12, \$24.

ONE of the problems of compiling a practical manual in molecular biology is to decide when to do it. The speed with which the subject progresses makes it difficult to judge when a method has progressed from research topic to established procedure. Perhaps the easiest way to identify areas that are ripe for such a treatment is to ask whether justification for use of the method has to be supplied with the protocol.

By this criterion, a number of the topics covered in Hames and Higgins's collection of articles were not ready for inclusion in a methods handbook. The point is most apparent in the first half of the book, which covers eukaryotic transcription and translation. Extensive protocols are provided in an annoying tabular form but they are heavily interspersed with results and discussion — this is particularly true of the chapter on expression of DNA in mammalian cells from Spandidos and Wilkie, who have otherwise written an interesting and extensive review of a rapidly developing field.

The second half of the book is devoted to

prokaryotic transcription-translation systems and eukaryotic *in vitro* methods and is a useful source of protocols, albeit in the same unsatisfactory format. Here Colman contributes a helpful and informative account of the use of *Xenopus* oocytes for translation of nucleic acids. Sandwiched between the two halves is a chapter on the use of *E. coli* polymerase to transcribe chromatin — a field which seems to me, even after reading this chapter, to have limited intellectual justification in view of extensive progress in *in vitro* techniques for faithful transcription of DNA by eukaryotic polymerases (well documented here by Manley).

It is worth comparing this volume with *Molecular Cloning: A Laboratory Manual*, published by Cold Spring Harbor Laboratory (reviewed in *Nature* 300, 782; 1982). The most striking difference is that in the latter book each method is presented clearly, without detailed discussion as to why one would want to make, for example, a phage lambda library. If the editors could have put together a manual on transcription and translation in a similar fashion, they would have produced a very worthwhile publication, but it is probably a few more years before this can be done. As it is the book contains several useful articles, but it is not one which I would buy for my laboratory. However, I think I would borrow it! □

Peter Little is a Staff Scientist at the Institute of Cancer Research, Chester Beatty Laboratories, London.

Cellular and other biologies

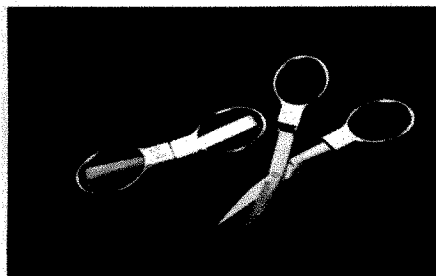
Many of the major laboratory equipment manufacturers are exhibiting at the current meeting of the American Society for Cell Biology.

● Newly introduced at the American Society for Cell Biology Meeting, the Dapple Systems, Inc. Video Image Measurement System (VIMS) performs direct on-screen measurement of features in video images produced by a camera mounted on a light or electron microscope or simply viewing a photograph. The video image and computer display are superimposed on a single monitor to allow marking of features. Measurement modes include outlining the periphery of features, tracing lengths, or making end points. Extensive statistical analysis programs report mean and variance, find comparisons between variables, and provide linear, log or polynomial fits. Distribution plots of one variable as a function of another are useful for studying correlations between two variables. The method of DeHoff and Rhines is employed to obtain distributions for the case of intersections of a planar surface through a solid with embedded features.

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● The Labconco RapidStill III is a compact automatic steam distillation unit used for nitrogen/protein determinations. Operation is completely automatic. After pressing the start button, the distillation process is controlled by a timer that determines the amount of water and sodium hydroxide used. One distillation takes less than 4 minutes. The distillation system automatically shuts down when the adjustable timer goes off.

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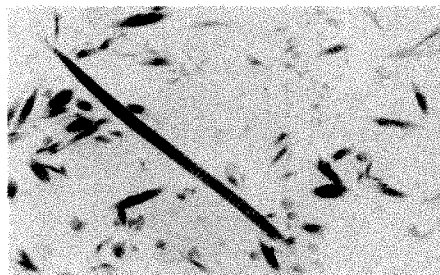
Fold-up scissors from B-J Scientific

● B-J Scientific Products of Albany, Oregon has introduced a compact, high-quality folding scissor called Slip-N-Snip, for use in the medical and scientific professions. These scissors are fabricated with stainless surgical steel blades and chrome-plated metal handles, and can actually cut through metal sheet with no damage to the blades. When not in use, Slip-N-Snip scissors can be easily folded up, with the blades completely shielded, and can be carried in a pocket with complete safety, unlike standard scissors.

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● RepliPlate is a new colony transfer pad for the rapid replica plating of bacterial colonies, from the BioProducts Department of Marine Colloids Division, FMC Corporation. Each pad is presterilized and ready-to-use. At least six precise replications can be made in a matter of seconds. RepliPlate pads simplify multiple-colony replication or library screening and are compatible with normal aseptic laboratory techniques. The pads are disposable to ensure biohazard containment and to prevent cross-examination.

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Electron micrograph of fibrillar collagen reconstituted from Vitrogen 100. Magnification $\times 30,000$.

● Vitrogen 100, the purified collagen for use in cell culture and biochemistry may now be purchased directly from Collagen Corporation of Palo Alto, California. Vitrogen 100 is a convenient, consistent and reproducible source of collagen which is economical to use. It is sterile, enzyme-solubilized bovine dermal collagen which has been extensively purified (99.9% pure), rigorously characterized and packaged for immediate use. Vitrogen 100 also eliminates problems with contaminants and batch-to-batch inconsistencies.

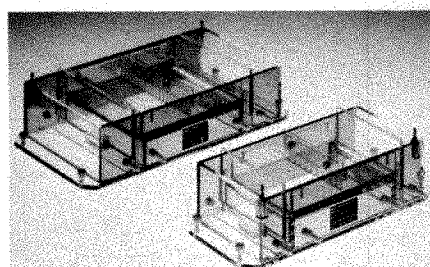
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● Pharmacia Fine Chemicals has introduced four new columns for high-performance preparative chromatography of proteins with sample loadings of up to 500 mg. The columns are based on the monodisperse ion exchangers Mono Q (anion exchanger) and Mono S (cation exchanger) with bed volumes of 8ml, Mono Q HR 10/10 and Mono S HR 10/10, or 20ml, Mono Q HR 16/10 and Mono S HR 16/10. Resolution is the same as is obtained in the well known columns, Mono Q HR 5/5 and Mono S HR 5/5, at sample loadings which are up to 20 times greater. Scale is direct with well described scaling factors which makes methods development straight forward and reliable. The columns are supplied for direct connection to the Pharmacia FPLC System. Accessories for hook-up to other HPLC systems are available.

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● ExpertVision, a new microcomputer-based system from Motion Analysis Corporation, allows the intricate movements of everything from microscopic organisms to human beings to robots to be analysed in detail. ExpertVision automatically converts images captured on videotape to a form that can be quickly analyzed by a computer. The system uses the video camera to capture images in real time. These analog video images are then played into a proprietary video processor that digitizes the image outline and inputs the data into a 16-bit, UNIX-based, high-resolution graphics workstation. The video processor displays the raw or processed images on the monitor, giving the operator immediate visual feedback. Once the digitized image is stored on the workstation's 42-megabyte hard disk, ExpertVision's software transforms the raw data into graphic images that depict the path of the motion. The digitized data are then analysed by a 16/32-bit microprocessor. The analysis can be displayed as numeric tabulated summaries, in graphic form (line graphs, bar charts, and three-dimensional images), or both. Hard-copy output of all screen displays can be generated on the system's printer/plotter.

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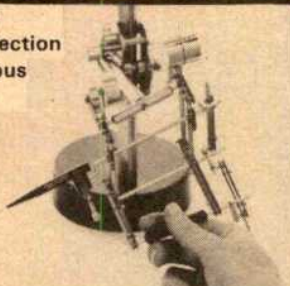
Electrophoresis for nucleic acids — American Bionuclear's two new systems.

● Designed primarily for nucleic acid analysis, the new American Bionuclear SHU-1000 (14 $\times$ 20 cm gel dimension) and SHU-2000 (20 $\times$ 20 cm) are versatile horizontal gel electrophoresis systems. Both models feature sliding gates adjacent to the end of the gel bed for pouring agarose gels in the removable ultraviolet transparent tray or directly onto the gel platform. An integral cooling chamber located beneath the gel bed ensures a constant gel temperature during runs. Ports on each reservoir permit recirculation of buffer to minimize changes in pH or ionic strength during separations. The SHU-1000 and SHU-2000 feature a levelling base and a choice of a wide variety of polycarbonate combs.

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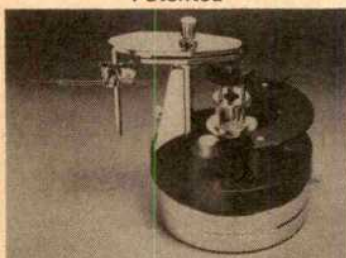
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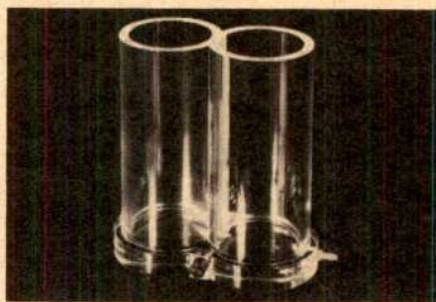
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● Laboratory Computer Systems, Inc. has added statistical analysis software to its Micro-Plan II image analysis system. The software is suitable for use with the IBM PC or PC/XT. The Micro Plan II is a low-cost, tablet-oriented image analyser that performs measurements such as length, area, centre of area, form factor, feret x-y, and maximum diameter of two-dimensional features traced on the digitizing tablet. The software allows the Micro-Plan II user to analyse these measurements statistically and to create data files in special formats that can be used for publication or read by Lotus Development Corporation's spreadsheet program 1-2-3. **Circle No. 108 on Reader Service Card.**

● Cambridge Isotope Laboratories is now offering carbon-13 labelled standards for the higher chlorinated homologue of the dioxin series. The particular standards are 1,2,3,4,7-pentachlorodibenzodioxin, 1,2,3,4,7,8-hexachlorodibenzodioxin, 1,2,3,4,7,9-heptachlorodibenzodioxin and 1,2,3,4,6,7,8,9-octachlorodibenzodioxin. Each standard is completely labelled with carbon-13 at an enrichment level of 99% or greater giving a net mass spectrometric response of + 12 AMU. Each standard is supplied at a specific concentration in nonane solution. The unlabelled standards of these same isomers are also available in standard solutions. Stable isotope labelled standards are already available for 2,3,7,8-tetrachlorodibenzodioxin and 1,2,3,4-tetrachlorodibenzodioxin. The addition of the penta through octa standards now makes it possible to do accurate GC/MS analyses on the whole series of most important dioxin isomers.

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Hoefler's new gradient makers.

● A recent addition to the Hoefler Scientific range of small gradient makers is a 500-ml model, the SG107S. The capacity of the SG107S makes it suitable for pouring ten or more 14 x 16 cm slab gels collectively in the Hoefler multiple casting chamber, the SE615. The SG107S can also be used for generating buffer gradients in column chromatography. Like the smaller Hoefler gradient makers, this one is made from sturdy acrylic. A simple-to-operate push-pull valve connects two cylindrical chambers which are mounted on a flat acrylic base. A side outlet port at the base of the mixing chamber may be connected to a pump to control the flow rate. Mixing is done on a magnetic stirrer.

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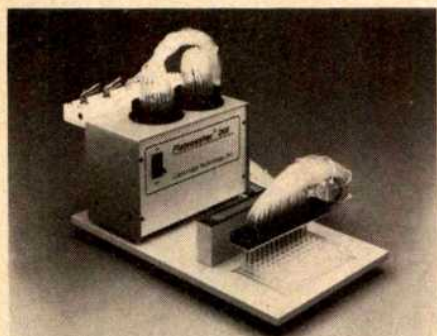
The LKB fraction collector for liquid chromatography.

● LKB-Produkter AB of Sweden has developed a new fraction collector, the 2212 HeliRac, for liquid chromatography. This microprocessor-controlled fraction collector uses circular racks. A wide range of tube sizes can be accommodated and three different collection modes (time, drop and pump signals) are available. Up to six time windows can be programmed for selective collection of peaks with known retention times. HeliRac has a rapid tube change time and is able to synchronize tube change with drop frequency to completely eliminate drops falling between tubes. The instrument case and keyboard are leakproof and resistant to most common solvents. Optional steel racks and a safety tray are available for the collection of aggressive solvents. An optional flow diverter can be used to divert unwanted eluent to a separate container. The collector can be used to control (via an interface) up to three pumps and three valves for automatic buffer changing.

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● The Packard Model 1700 sample processor is a programmable digital diluter with a wide variety of applications. The two modules, diluter and keyboard, perform functions which include pipetting, diluting dispensing and multiple dispensing, sample only transferring, sample aliquoting, serial diluting and titrating. The diluter is driven by a Z-80 microprocessor and can communicate with other instruments via its RS232 port found on the back of the instrument. The keyboard, one 12-button keypad and four command keys, allow the user to communicate with the diluter. A touch-sensitive membrane overlay protects the keyboard from chemical spills. An eight character alphanumeric vacuum fluorescent display shows program information, memory location, and error codes. The movable keyboard module is attached to the sample processor by a flexible cord and can be positioned for the operator's convenience. The instrument will accommodate syringes of various sizes to maximize flexibility. The aspirate syringe pipettes sample or reagent, and the reagent syringe draws in the reagent that is to be dispensed.

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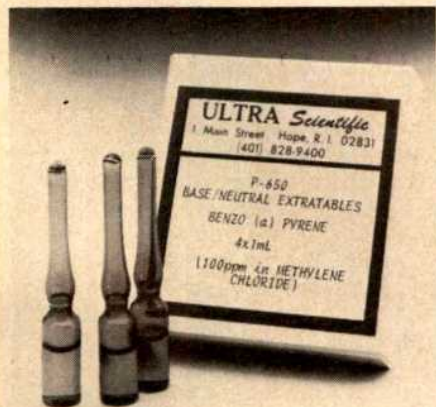
Enzyme-linked immunosorbent assay plates can be washed on the Model 260 from Cambridge Technology.

● The Model 260 Platewasher from Cambridge Technology, Inc. of Cambridge, Massachusetts is now available with an optional wash manifold that permits as many as four different wash fluids to be used with the instrument without requiring manual change of tubing connections. The Model 261 manifold is constructed of high density polyethylene. No lubrication is required and the materials are compatible with most common wash fluids such as water, saline, alcohols, and trichloroacetic acid. The Model 260 Platewasher is designed to be used with EIA/ELISA procedures. It is a semi-automated system for washing the wells of microtiteration plates. The flow to each of the 24 wash needles is controlled individually and simultaneously, providing high well-to-well uniformity and making the unit much faster than manual techniques.

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● Ultra-pure standards and standard mixtures for performing EPA 600 methods by gas chromatographic analysis are now available from Ultra Scientific. Each kit contains 1ml of each standard required for the particular method plus 1ml of a mixture. Individual standards are supplied in sealed 1ml ampules. Standards are prepared in solution, ready to use and guaranteed to specification. No additional preparation or handling of these hazardous materials is required. Free samples of standard mixtures for any EPA method from 600 to 612 are available on request from Ultra Scientific.

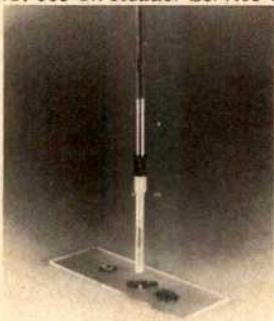
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Ultra's new standards.

● Du Pont's Prep Type OD cartridges for the automated solid phase extraction of lipophilic components from aqueous samples are designed to be used with the Du Pont Prep automated sample processor. The Type OD cartridge partitions components between a moving liquid phase of buffered aqueous sample matrix, wash solvent, or eluting solvent and a solid stationary phase of resin bed. Both aqueous and organic solvents may be used to extract lipophilic analytes from aqueous solutions. Each cartridge comprises a cap, a 4 ml extraction column, a 6 ml effluent cup, and 4 ml recovery cup. Du Pont also provides a method development guide to aid extraction of lipophilic analytes.

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● The MI-720 micro-carbon dioxide electrode is claimed to be the smallest commercially available CO₂ electrode capable of measuring CO₂ in small volumes and droplets. Its tip with membrane attached is 3mm in diameter and its total length is 6.2cm. Its useful concentration range is 10⁻² to 10⁻⁴ (440 to 4.4 p.p.m.) CO₂. The MI-720 is one of a range of "small volume" electrodes from Contact Microelectronics, Inc. of Londonderry, New Hampshire.

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From Houston Instruments/Bausch & Lomb, the 4500 MicroScribe.

● The Series 4500 MicroScribe strip chart recorder from Houston Instruments is one of a new generation of recorders using microprocessor control and touch-panel switching. The recorder's centralized liquid crystal display provides constant information about operating parameters as well as self-test messages such as 'paper out' or voltage 'out of range'. Charts from the Series 4500 span a full 20 cm, at speeds ranging from 1 cm per hour to 60 cm per minute. Linearity is within 1% full scale; response is 0.25 seconds for full-scale.

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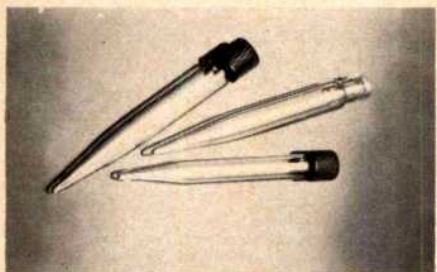
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● A new restriction enzyme, *Asp718*, is now available exclusively from Boehringer Mannheim Biochemicals. This enzyme recognizes the same sequence as *KpnI*, GGATACC, but produces 5' protruding cohesive ends, instead of the 3' ends produced by *KpnI*. This enzyme has one recognition site in simian virus 40 DNA, but no sites in pBR322 or 328 DNA.

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● Spectra-Physics' new liquid chromatography sampling system, the SP8780XR automatic sampler, is a stand-alone module which automates any modular liquid chromatography system, while also providing fully integrated operation with other Spectra-Physics LC modules. Control and communication are provided via the company's local area network, LABNET, RS-232-C, and contact closure circuits. A novel feature of the SP8780XR is the ability to use bar code labels on each individual sample vial. The bar codes provide each vial with a unique identification to aid in sample tracking and eliminate errors in tray loading. The code also carries analysis file information for each vial, thereby automating instrument setup in LABNET systems.

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● A new bench-top liquid scintillation counter from Beckman, the LS1801, is a three-channel system featuring a rack sample changer that will accommodate either standard vials (336 samples) or miniature vials (648 samples). Beckman's Versa-Rack sample changer allows both types of vial to be used in the same instrument without the need for adapters. An exclusive feature on Beckman counters is the Horrocks number method of quench monitoring. One quench curve generated using *H* number will provide accurate results, regardless of the volume of sample, type or vial or choice of cocktail. The LS 1801 can carry out microvolume counting with a sample/cocktail volume as low as 200 μ l, thus cutting consumable costs.

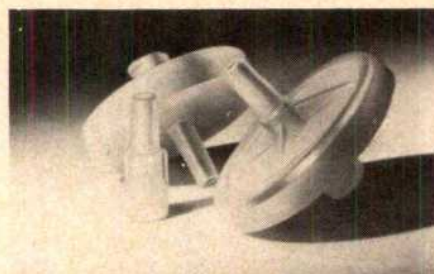
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● Intended for use with the Flow Titertek 8- or 12-nozzle pipettors, the new Ejector Bars produced by Robbins Scientific guarantee ejection of every tip, every time. Made of durable, solid aluminium, they can be attached quickly and easily to Titertek pipettors. They replace existing bars with no pipettor modification required.

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● Normal- and reverse-phase silica media for liquid chromatography have been added to the Amicon Corporation range. Matrex silica chromatographic media are available in a wide range of particle sizes, with pore sizes ranging from 60 to 250 Å. All may be ordered with C18 or C8 functionality for reverse-phase applications. Equivalent capacity and selectivity factors for all particle sizes are especially valuable for direct scale-up to large preparative applications, avoiding the necessity of method redevelopment.

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Spartan filters for use with syringes.

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● The 1984 Product Reference Guide from Pharmacia P-L Biochemicals, which lists products for biochemistry and molecular biology, is now available. The guide is divided into sections based on product categories which include: Enzymes, Restriction nucleases, Nucleic acids for molecular biology, Polynucleotides, Nucleotides, Coenzymes, Affinity chromatography, Antibiotics, Buffers, Carbohydrates, Immunologicals, Lectins, Lipids and Phorbol esters.

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● New from BBL is an inorganic iodine stain product to improve Gram staining. The new iodine offers an alternative to breakage of glass ampoules to prepare a working iodine solution. The BBL Gram's iodine with concentrate is packaged in component sets containing four 250-ml polypropylene bottles of diluent with four 25-ml bottles of iodine concentrate. As with other BBL Gram stain reagents, the new product features a flip-top cap, compact tray/work station and stain resistant labels. The two bottles mix readily to prepare the working iodine solution. The current BBL stabilized PVP iodine products (in kits, sets or gallon form) are still available to microbiologists who prefer the elimination of the working solution preparation step and a longer stability.

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● Oligonucleotide hybridization probes of extremely high specific activity have been used successfully to detect many different genes by hybridization analysis. A new technical information bulletin (Bulletin T-1588) from Beckman Instruments, Inc. shows how to use the Model 170 radioisotope detector to automate the sample handling of the preparation of labelled oligonucleotides to minimize radiation exposure. The detector can be used for desalting labelled oligonucleotides, minimizing the exposure to large amounts of radiation and providing analog records of the run in addition to integration data that enables easy calculation of the specific activity.

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● A new addition to Vector Laboratories biotin/avidin system line of products is the amplifying agent biotinylated anti-avidin D. This molecule is capable of binding to avidin D via its biotin residues as well as through conventional antibody-antigen interactions. The potential for multiple-site binding appears to provide this antibody with unusual characteristics. When used in conjunction with fluorescent or enzyme-conjugated avidin D, a large amplification of the primary signal can be achieved. This reagent can be used with biotinylated DNA hybridization probes, hormones, toxins, antibodies or lectins to enhance the desired signal.

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These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.

Awards

Twenty-nine research fellowships have been awarded under the European Science Exchange Programme as follows: **Ms Victoria J. Allan**, Department of Biology, University of York, to spend one year from February 1985 at the European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany; **Mr Ilesh Bidd**, School of Chemistry, University of Bristol, to spend one year from January 1985 at the Institut de Chimie, Université Louis Pasteur de Strasbourg, France; **Mr Alexander W. Blayney**, University Hospital of Wales, Cardiff, to spend one year from January 1985 at the department of otolaryngology, University of Bordeaux, France; **Mr David H. Burgess**, School of Mathematical Sciences, Queen Mary College, London, to spend one year from January 1985 at the section d'astrophysique, Observatoire de Paris, Meudon, France; **Mr I. Colin G. Campbell**, Department of Mathematics, King's College, London, to spend one year from January 1985 at the Institute of Theoretical Physics, University of Stockholm, Sweden; **Mr David B. Carpenter**, Department of Physics, University of Edinburgh, to spend nine months from January 1985 at the Deutsches Elektronen-Synchrotron, Hamburg, Federal Republic of Germany; **Mr Christopher Carr**, Department of Inorganic Chemistry, University of Bristol, to spend one year from January 1985 at the Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm, Sweden; **Mr Christopher L. Clayton**, Department of Applied Biology, University of Wales Institute of Science and Technology (UWIST), Cardiff, to spend one year from January 1985 at the Department of Medical Biochemistry, University of Geneva, Switzerland; **Miss Margaret E. Collins**, Department of Botany and Microbiology, University College, Cardiff, to spend one year from January 1985 at the Institut de Génétique Moléculaire, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France; **Mr George M. Coupland**, Department of Molecular Biology, University of Edinburgh to spend one year from January 1985 at the Institut für Genetik, University of Cologne, Federal Republic of Germany; **Mr Irwin B. Davidson**, MRC Institute of Virology, University of Glasgow, to spend one year from March 1985 at the Laboratoire de Génétique moléculaire des Eucaryotes, Centre National de la Recherche Scientifique, Strasbourg, France; **Mr Christopher S. Durbin**, School of Geography, University of Manchester, to spend one year from July or September 1985 at the Laboratoire de Géomorphologie, Université Libre de Bruxelles, Belgium; **Mr John L. Edwards**, School of Chemistry, University of Bristol, to spend one year from January 1985 at the

Collège de France, Paris, Francis; **Dr Catherine W. Gill**, Marine Science Laboratories, University College of North Wales, Menai Bridge, to spend one year from October 1984 at the Station Marine de Roscoff, France; **Mr David R. Greaves**, Biophysics Department, King's College, London, to spend one year from January 1985 at the Netherlands Cancer Institute, Amsterdam, the Netherlands; **Miss Kim E. Hammond**, School of Biological Sciences, University of East Anglia, Norwich, to spend one year from January 1985 at the department of phytopathology, Agricultural University, Wageningen, the Netherlands; **Dr Barbara J. Hart**, Department of Zoology, University of Glasgow, to spend one year from September 1984 at the Institut Royal des Sciences Naturelles de Belgique, Brussels; **Miss Elaine J. Hughson**, Department of Haematology, Royal Hallamshire Hospital, Sheffield, to spend one year from October 1984 at the European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany; **Mr Benjamin P. Jeffries**, Mathematical Institute, University of Oxford, to spend one year from January 1985 at the Max Planck Institute for Physics and Astrophysics, Munich, Federal Republic of Germany; **Dr Deborah J. Jones**, Department of Chemistry, University of Southampton, to spend one year from 1 October 1984 at the Laboratoire des Acides Minéraux, Université des Sciences et Technique du Languedoc, Montpellier, France; **Dr Peter J. McDonald**, Department of Physics, University of Nottingham, to spend one year from January 1985 at the Service de Physique du Solide et de Résonance Magnétique, Saclay, France; **Dr Janice G. Mather**, Department of Zoology, University of Manchester, to spend one year from October 1984 at the Institute of Zoology and Zoophysiology, University of Aarhus, Denmark; **Mr Ronald A. Milligan**, Department of Structural Biology, Stanford University School of Medicine, California, USA, to spend one year from April 1985 at the European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany; **Dr Jonathan Mitchley**, Botany School, University of Cambridge, to spend one year from January 1985 at the Department of Plant Ecology, University of Utrecht, the Netherlands; **Mr Michael J. Morris**, School of Chemistry, University of Bristol, to spend one year from January 1985 at the Institut de Chimie, Université Louis Pasteur, Strasbourg, France; **Mr Mark A. Riley**, Department of Physics, University of Liverpool, to spend one year from October 1984 at the Niels Bohr Institute, University of Copenhagen, Denmark; **Mr Andrew Robinson**, Department of Astronomy, University of Manchester, to spend one year from January 1985 at the European Southern Observatory, Garching bei München, Federal Republic

of Germany; **Mr Robin G. Stuart**, Clarendon Laboratory, University of Oxford, to spend one year from May 1985 at the theory division, CERN, Geneva, Switzerland; **Dr Simon G. Webster**, School of Animal Biology, University College of North Wales, Bangor, to spend one year from October 1984 at the Institut für Zoophysiology, Rheinische Friedrich-Wilhelms-Universität, Bonn, Federal Republic of Germany.

The Royal Society of Chemistry has announced a cycle of new awards for British and Commonwealth citizens sponsored by various industrial companies; separate prizes are available to scientists working in the fields of: Analytical Separation Methods; Chemical Analysis and Instrumentation; Chemical Education; Health Safety or Environmental Chemistry; Hydrocarbon Oxidation Chemistry; Main Group Element Chemistry; Natural Product Chemistry; Organic Reaction Mechanisms; Solids and Materials Processing; Spectroscopy; Structural Chemistry; and Thermodynamics. Awards will be available for a different range of subjects in subsequent years. Further details from Mrs Y.A. Fish, The Royal Society of Chemistry, Burlington House, London W1V 0BN, UK.

The first in a series of scientific research funding awards, worth over £380,000 a year has been announced by the Wellcome Trust, Britain's largest medical charity, in association with the Royal Australasian College of Physicians. The first two Australian Wellcome Senior Research Fellows will be **Dr. David Healy** of the Prince Henry's Hospital, Melbourne, who will work on hormone activity in pregnancy during his studies on endocrinology in obstetrics and gynaecology, and **Dr Keryn Williams** who will be investigating the problems of rejection in corneal transplants at Flinders University, Adelaide.

To celebrate one hundred years of publication of Baillière's Clinical Pharmacology, **Baillière Tindall** is sponsoring an essay competition for medical and pharmacy undergraduates. There is a first prize of \$1,000 and two second prizes of \$500; further information is available from The Editorial Director, Baillière Tindall, 1 St Anne's Road, Eastbourne, East Sussex BN21 3UN, UK. The Royal Swedish Academy of Sciences has awarded the **Crafoord Prize** for ecological research to **Professor Danial H. Janzen**, a Professor at the University of Pennsylvania, USA, for his studies on co-evolution.

A major European Conservation Award scheme has been announced by the **Ford Motor Company**, operating in eight European countries: Austria, Holland, Belgium, Italy, Spain, Switzerland, France, and the United Kingdom. Total funds exceed \$100,000 and projects can be submitted in up to six different categories

including rural, urban and heritage conservation. Further information can be obtained from The Conservation Foundation, 11a West Halkin Street, London, SW1X 8JL, UK.

The International Agency for Research on Cancer is inviting applications for its Training Fellowships and a Visiting Scientist Award for 1985/86. Further details obtainable from the Chairman of the Fellowships Selection Committee, International Agency for Research on Cancer, 150 Courts Albert-Thomas, 69372 Lyon Cedex 08, France.

Pool Bioanalysis Italiana, is inviting entries of scientific papers on environmental microbiology in their first international competition; the prize is one week's holiday in Rome. Any paper in which SAS, SAS Compact or MTM-3 air samplers have been used is acceptable. For further details write to "P.B.I.", 1st International Competition, Via Novara 89, 20153 Milano, Italy.

Nominations are now open for the 1985 **Lita Annenberg Hazen Awards for Excellence in Clinical Research**, the purpose of which is to encourage increased participation in clinical research by physicians. Prizes amounting to \$100,000 are available and further details can be obtained from James F. Glenn, M.D., President, The Mount Sinai Medical Centre, Chairman, The Lita Annenberg Hazen Awards Program, 1 Gustave L. Levy Place, New York, New York 10029, USA.

The **Smithsonian Institution** award post-doctoral, predoctoral and graduate research fellowships in the fields of *inter alia*, the History of Science and Technology, Earth Sciences, Anthropology, and Biological Sciences. For details and application forms contact the Office of Fellowships and Grants, Desk J, 3300 L'Enfant Plaza, Smithsonian Institution, Washington, D.C. 20560, USA.

Appointments

Dr Denver Hall has been appointed to the Unilever Chair in Colloid and Surface Chemistry at the University of Salford.

Professor Barry R. Jennings of the University of Brunel has been appointed as the new Professor of Physics at the University of Reading.

Meetings

14 November, **Meeting on the Use of Physical and Analytical Techniques in Drugs Research**, London (Conference Secretariat, Society of Chemical Industry, 14 Belgrave Square, London SW1X 8PS, UK).

22-23 November, **Clonal Evolution of Tumours**, British Association for Cancer Research/Royal Society of Medicine Joint Winter Meeting, London (Barbara Cavilla,

BACR, c/o Institute of Biology, 20 Queensberry Place, London SW7 2DZ, UK).

29 November, **One Day Symposium on Food Production and Our Rural Environment — The Way Ahead**, London (Centre for Agricultural Strategy, University of Reading, 1 Early Gate, Reading RG6 2AT, UK).

4-6 December, **8th International Online Information Meeting**, London (Learned Information (Europe) Ltd, Besselsleigh Road, Abingdon, Oxford OX13 6LG, UK).

25 February-1 March, **36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy**, New Orleans, USA (Mr David R. Weill III, 437 Donald Road, Pittsburgh, Pennsylvania 15235, USA).

25 February-1 March, **International Conference on Climatic, Biotic and Human Interactions in the Humid Tropics**, Sao Jose dos Campos, Brazil (Mr Walter Shearer, Senior Programme Officer, Development Studies Division, The United Nations University, Toho Seimei Building, 15-1, Shibuya 2-chome, Shibuya-ku, Tokyo 150, Japan).

25-28 March, **2nd Conference of the International Association for Breast Cancer Research**, London (Dr Marvin Rich, AMC, Cancer Research Center, 6401 West Colfax Avenue, Lakewood, Colorado 80214, USA).

1-4 April, **3rd International Meeting on Low Temperature Microscopy and Biological Analysis** Cambridge, UK (The Administrator, Royal Microscopical Society, 37/38 St. Clements, Oxford OX4 1AJ, UK).

3-4 April, **21st Annual Meeting of the National Council on Radiation Protection and Measurements**, Washington D.C., USA (Mr Roger Ney, NRC, 7910 Woodmont Avenue, Suite 1016, Bethesda, Maryland 20814, USA).

10-12 April, **International Symposium on Chemistry and Physics of Baking: Materials, Processes and Products**, Sutton Bonington, Leicestershire, UK (Mr J.M.V. Blanshard, Department of Applied Biochemistry and Food Science, The University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough, LE12 5RD, UK).

16-18 April, **International Symposium on the Impact of Biotechnology on Diagnostics**, Rome, Italy (Organizing Secretariat, Fondazione Giovanni Lorenzi, Via Montenapoleone 23, Milan, Italy).

16-18 April, **Conference on Advanced Computational Techniques for Information Retrieval**, Oxford, UK (The Association for Information Management, 3 Belgrave Square, London SW1X 8PL, UK).

23-24 April, **Meeting on Material Properties and Stress Analysis in Biomechanics**, Brunel University, UK (The Institute of Physics, Meetings Office, 47 Belgrave Square, London SW1 8QX, UK).

27 April-2 May, **Congress on Laboratory Medicine**, Hamburg, FRG (Dr O. Fenner, Organizing Committee, Kongress für Laboratoriumsmedizin, Hamburg Messe und Congress GMBH, Ausstellungsabteilung, Postfach 30 24 80, D-2000 Hamburg 36, FRG).

28 April-1 May, **International Conference on Arctic Water Pollution Research: Applications of Science and Technology**, Yellowknife, Northwest Territories, Canada (Conference Services Office, National Research Council of Canada, Montreal Road Laboratories, Ottawa K1A 0R6, Canada).

28 April-4 May, **21st EUCHEM Conference on Stereochemistry**, Bürgenstock, Switzerland (Professor M. Julia, Laboratoire de Chimie, E.N.S., 24 rue l'homond, 75231 Paris Cedex-05, France).

29 April-2 May, **23rd International Magnetics Conference**, St. Paul, Minnesota, USA (Mr J.A. Nyenhuis, School of Electrical Engineering, Purdue University, West Lafayette, Indiana 47907, USA).

30 April-3 May, **Conference on Computer Aided Production Management**, Wembley, Middlesex, UK (The Manager — Conferences and Exhibitions, The Institution of Production Engineers, Rochester House, 66 Little Ealing Lane, London W5 4XX, UK).

7 May, **37th International Symposium on Crop Protection**, Ghent, Belgium (The Secretary, Faculteit Van de Landbouwwetenschappen, Rijksuniversiteit, Coupure Links, B-9000 Gent, Belgium).

9-10 May, **International Symposium on Thyroid Function Tests**, Ile de Bendor, France (The Secretariat, Laboratoire des Hormones Proteiques, Faculté de Médecine, F-13385 Marseille Cedex 5, France).

21-23 May, **Biotech 85 Europe**, Geneva, Switzerland (Online Conferences Ltd, Pinner Green House, Pinner, Middlesex, HA5 2AE, UK).

21-23 May, **7th Annual Symposium on Safeguards and Nuclear Material Management** Liège, Belgium (Mr L. Stanchi, CEE-JRC, I-21020 Ispra Varese, Italy).

22-24 May, **International Congress on Tissue Integration in Oral and Maxillo-Facial Reconstruction**, Brussels, Belgium (Dr D. van Steenberghe, Faculty of Medicine, Catholic University Leuven, Kapucijnenvoer 7, B-3000 Leuven, Belgium).

3-7 June, **International Symposium on Vaccines and Vaccinations**, Paris, France (Secretariat, Symposium on Vaccine and Vaccinations 1985, Institut Pasteur, 28 Rue du Docteur Roux — 75724 Paris Cedex 15, France).

4-6 June, **International Seminar on Electronics and Traffic on Major Roads**, Paris, France (Secretariat of the Seminar on Electronics and Traffic, European Conference of Ministers of Transport, 19 rue de Franqueville, 75775 Paris Cedex 16, France).

What will happen to UNESCO?

The British Government seems about to follow the United States into resignation from UNESCO. That will not be the end of the world (or even of UNESCO).

THE next few weeks will decide whether the UN Educational, Scientific and Cultural Organization (UNESCO) will survive in its present shape into the 1990s. And the outlook is far from bright. There now seems very little chance that the United States will go back on its decision of a year ago to pull out six weeks from now. The State Department's demand that the UNESCO administration should mend its ways has been partly met, but not sufficiently to persuade a president temperamentally biased towards withdrawal to change his mind. And there seems to be a strong constituency, among UNESCO's users in the United States, that last year's decision should be unscrambled (see p.300).

UNESCO's problem of managing without a quarter of its budget has now been further complicated by the chance that the United Kingdom will also, in the next few weeks, give notice of withdrawal. Such a decision, on the cards since the stern letter to UNESCO from the British government earlier in the year, has been made more likely by the tightness of the budget on which the Foreign and Commonwealth Office will have to live in the next three years. Even UNESCO's supporters in Britain, those who have held that £5 million a year is a small price to pay for continued membership of an organization from which withdrawal is bound to give widespread offence, will think differently when they recognize that the UNESCO subscription can be paid only at the expense of other useful activities — those of the British Council (the United Kingdom's own UNESCO), of the overseas broadcasts of the British Broadcasting Corporation or of direct foreign aid (for example to Ethiopia). So it is likely that the British government, anxious to clear its collective desk before the end of the year, will write a letter giving notice of withdrawal, put it in the mail and hope that the fuss will be dissipated during the coming holidays.

But what fuss? The obvious risks of withdrawal from UNESCO are political. To pull out of one UN organization is to put membership of all others in doubt, inviting the complaint that loyalty to international causes has been undermined. Because UNESCO has become widely regarded by the governments of developing countries as one of the chief vehicles for the transfer of the skills of advanced societies to those that lack them, those that withdraw will run the further risk of being thought indifferent to the cause of development. And because the row within UNESCO has been closely linked with personal criticism of the present director general, Mr Amadou-Mahtar M'Bow, enthusiastically the choice of the non-aligned countries on his appointment ten years ago, pulling out is bound to seem a slap in the face to those who voted for him in the first place.

Justification

The United States government is better able to meet these complaints head on than is the British; it can always point to the scale of its financial contributions to international organizations

of all kinds, the International Monetary Fund and the World Bank as well as the United Nations system. The British government has less to boast about, and is politically involved with many of UNESCO's fondest friends through the machinery of the British Commonwealth. If Britain does in the end withdraw, it had better pay close attention to the way in which its decision is justified.

Many of the complaints against UNESCO in the past several years are beside the point. Both the US and British governments say that UNESCO, with its remit to consider matters educational, scientific and cultural, should not be caught up in the political arguments that have dominated its general assemblies in recent years. But that is to ask too much. An organization whose terms of reference give it a proper interest in the care of the cultural heritage (and which has done valuable work in safeguarding the monuments along the Nile) cannot, for example, avoid also giving Greece an opportunity for attacking the British government because the Elgin marbles, which once adorned the Parthenon, are still on show in the British Museum. Similarly, it has from the start been inevitable that UNESCO should have been a battleground for conflicting interests over issues such as the control of copyright on publications (and the 1971 Paris amendment to the Berne Convention on copyright is both a feather in UNESCO's cap and a considerable victory for developing countries within UNESCO).

Given UNESCO's membership and constitution, such issues have been unavoidable from the start. Even Mr M'Bow's "new information order", a set of proposals whose immediate effects on the international news gathering agencies would have been disastrous, and which would also have legitimized the ambitions of governments to control what is said about them, was a proper question for UNESCO to consider. The complaint against UNESCO, and its management in particular, is that it seems not to have wished to lift a finger to avoid irreconcilable conflicts on these issues. Indeed, it has often seemed to relish them.

Blame

For this state of affairs, the developing countries must shoulder a substantial part of the blame. UNESCO is not by its constitution a supranational authority whose decisions are binding on its members, but rather a forum in which countries with something that others want may be persuaded to offer it. This is what happened with the revision of copyright protection, an international convention in whose negotiation UNESCO acted as a midwife, by which governments of developing countries were given limited rights to use educational materials if the original publishers are unable or unwilling to make them available. The difficulty that has arisen in the past few years is that the principle of one government, one vote, has gone to the heads of a majority of the membership. Arguments about issues such as the new information order are one thing (and should be tolerable), but proposals to spend a substantial part of communal funds supplied chiefly by those opposed to the proposal are another. One of the changes at UNESCO for which the United States has been pushing in the past year is the replacement of the present voting procedure by one in which those who pay the most have the most influence, but that would have been a cosmetic device for burying conflict, not for creating the atmosphere of consensus without

Science in the Soviet Union

THE special issue of *Nature* surveying science in the Soviet Union has been delayed until next month. □

which any communal fund must be misspent.

But why has the management allowed things to come to such a pass? That is a question that may always be fairly asked when a voluntary organization is in danger of being torn apart by internal conflicts. The usual answer is that the management has failed to spot trouble coming, and to head it off. That complaint unquestionably applies to UNESCO's management in the past few years. Mr M'Bow has been most often criticized, during UNESCO's troubled two years, for his tolerance of inefficiency, for his dictatorial ways with UNESCO's staff, for nepotism and because of his undoubted vanity. But the failing that matters is that he has not been able, or has not chosen, to function as the diplomat an organization such as UNESCO needs. Whatever members depart from UNESCO in the next few weeks, there is the strongest possible reason why Mr M'Bow should also quit — or be asked to go. UNESCO will be a substantial organization even if Britain follows the United States (and is followed by other members) into the cold. Especially with a shrunken budget, there is the strongest reason why UNESCO should be managed well.

Inevitably, it goes against the grain for governments wedded to the doctrine that it is injudicious (to say the least) to attack people and not policies to tell Mr M'Bow outright that he is an encumbrance to the organization he purports to head. Even so, it is mystifying that Mr George Shultz, when giving notice a year ago of the United States' intentions to withdraw, should have gone out of his way to emphasize that Mr M'Bow's misfortune is that he happens to have led UNESCO from a state of inconspicuous mediocrity to one of conspicuous failure. He would have been better served if the member governments now bent on withdrawing had told him sooner, and to his face, what they were always eager to tell others privately. He may now fairly complain that he was set up. But he has been a failure nevertheless.

By now, the principles on which reform should be sought are plain. Since it began, UNESCO has been wrongly organized for what it is now hoping to accomplish, the spending of a relatively modest \$200 million a year on good works scattered about the surface of the Earth. In effect, it is a modestly-sized charitable foundation whose terms of reference are international, but it is organized not for spending money efficiently but as if it were the kernel of an evolving academy of all the talents. The result is that the members of its staff are never sure whether they have been appointed in their capacity as scholars or as people who can channel funds to others who will be productive. And there are too many of them, by any set of job-descriptions, perhaps by an order of magnitude. It is laughably outrageous that UNESCO should spend two-thirds of its income on itself. When the present alarm about withdrawal, intended or merely threatened, is over, the remaining members should set about putting things to rights. The French government should be well placed to take the initiative.

Determination

What needs to be done is simply told. Inefficiency apart, UNESCO differs from other organizations. Ideally, its role should be to sponsor international intellectual projects that others have not attempted, being prepared to see many of these fail but resolute in its determination that those that succeed should be launched independently, with other people's funds, once their success is apparent. Instead, UNESCO has more often waited for other people's band-wagons to pass, seeking a small share in their sponsorship but a larger share of the credit. This, for example has been UNESCO's stance during the past quarter of a century in respect of the improvement of science teaching in secondary schools, where great opportunities have been missed for translating internationally programmes that have functioned well in member states. By contrast, UNESCO has embraced with too much enthusiasm schemes for teaching what is loosely called "peace" to unsuspecting teenagers throughout the world in circumstances when the most sophisticated educational systems are unable to provide models on which to practise what is being preached.

The conventional assumption is that none of these criticisms applies to UNESCO's scientific work, but even that claim is

shaky. It is, however, true that UNESCO is at present the conduit for continuing support for some important institutions, the International Council of Scientific Unions (ICSU) and the International Centre for Theoretical Physics (at Trieste), originally supported by the International Atomic Energy Agency. There is the strongest case why such institutions should not collapse, and why members thinking of withdrawing from UNESCO should ensure that their contributions to these central organizations should continue. UNESCO has also become the umbrella for a number of important coordinating research committees, the best known and most useful in oceanography. Thus the organization provides house-room and a secretariat for the International Oceanographic Commission, and also gathers oceanographic data collected by merchant vessels on routine voyages — tasks not at present done by other means. But UNESCO happens to be responsible only by accident, because it was the first to take on these tasks. As things have turned out, it would be more appropriate that such jobs should be done by ICSU or one of its member councils — and that the practical task of data-storage should be turned over to suitable laboratories working to contracts.

What might have been UNESCO's pride and joy in the past few years, however, the Man and Biosphere Programme (which is really an umbrella for a host of coordinated ecological projects scattered over the surface of the Earth, separately financed by national governments), is one to which the management in Paris has devoted much less enthusiasm than the case demands. But there is enough merit in UNESCO's scientific programme for the price of withdrawal from the organization to be an undertaking that the deserving parts of the programme should not be harmed.

Earlier this year, a committee of the US National Academy of Sciences under Walter A. Rosenblith of Massachusetts Institute of Technology made just this point to the State Department. Will the British government be prepared to follow suit? Its first reaction will be to say that if there is a chance of saving £5 million a year, it would prefer to keep the lot for other deserving causes. But this would be unacceptable, at least if worthwhile international institutions are to be endangered. Another unavoidable question is that of whether ICSU will be able to shoulder the extra burdens likely to be thrust on it even if the United States is the only member to pull out of UNESCO. The answer is that, at present, ICSU as such is not equipped to do much more than organize the committees necessary to ensure its continuing existence, but that situation need not persist. Indeed, there is a strong and quite separate case why, in the interests of the international community, ICSU should now be strengthened, and equipped with the resources necessary for it to play a stronger executive role in international science. But there is an obvious danger in supposing that ICSU might become a partial substitute for UNESCO — that ICSU might in the process itself be damaged, and that in any case there is no escaping some of the political difficulties that have made UNESCO uncomfortable in the past few years.

Reorganization

How, in these circumstances, should the British government decide? The question is not black and white. Pulling out regardless of the consequences would be foolish (and is unlikely). Pulling out without being prepared to pick up the pieces would similarly be wrong. Giving notice of withdrawal on the vague understanding that the decision might be reversed if UNESCO mends its ways would be fruitless, as the experience of the United States in the past year has shown. The best course is to couple a declaration of an intention to withdraw (not necessarily a year from now) with a prospectus describing how UNESCO might be organized efficiently, and how its reduced budget might nevertheless be spent effectively. The trouble, in the past year's bickering, is that it has concentrated on attempts to reform a well-intentioned organization absurdly ill-suited to its purposes by tinkering with the administration. What UNESCO needs is not to be told repeatedly why it is absurd, but to be helped to understand what it is for, and how it might function well. □

Fifth generation computers

Japan finds rivals treading uncomfortably close

Tokyo

JUDGING by the scale of Western governments' reactions to the fifth generation computer project, described by representatives to the project's international conference which ended in Tokyo last week, Japanese officials must be wondering if they would not have been better off keeping quiet about the whole subject.

Just three years ago, the project was launched in a storm of hyperbole that made it seem that Japan was about to seize world computer markets through a qualitative leap forward into a new world of super-intelligent computing systems. Now, Japan no longer looks the world leader as tough competing projects launched by the United Kingdom, the United States, France, Germany and the European Communities take off. Nor, despite earlier hopeful discussions between the United Kingdom and Japan, does there seem to be any longer a significant chance that cooperation will replace competition.

Strangely, the project that began it all, Professor Kazuhiro Fuchi's fifth generation computer project run from the Institute for New Generation Computing (ICOT), now looks academic and narrow, despite having been originally branded a "computing apocalypse" that would destroy Western nations' information technology industries. The explanation is Fuchi's continuing belief that the next phase of computer history will come from computers that use predicate logic to carry out inferences at high speed.

The language PROLOG, specially designed to handle such operations (see *Nature* 309, 198; 1984) has been taken as the project's starting point. With an inference mechanism at its core, the fifth generation project will try to use high speed mechanisms for retrieval from massive knowledge bases, permitting the computer to function as a highly intelligent "expert" system, while intelligent interfaces allow users to speak to their computers.

The intended results, very high speed inference and knowledge retrieval and intelligent man-machine interfaces, are what all the competing nations' projects aim at. But not everyone believes that predicate logic — and PROLOG — is the way to achieve them. As Daniel Bobrow of Xerox's Intelligent Systems Laboratory put it, "no single paradigm is appropriate to all problems. Just as there are many tools in a carpenter's tool box, there must be many in the programmer's kit." Fuchi sees many other approaches as being subsumed by logic programming (not to be confused with logical programming). If he

is right, his institute may well be way out in front in a couple of years; if not it could be left trying to "pry up nails with a screw-driver".

So far, anyway, ICOT has delivered on its promises. A working sequential inference machine was on display at the conference and will provide the essential "programming environment" (essentially equivalent to a laboratory bench and equipment) for further research. But from now on, the really hard problems begin — particularly that of parallel processing — where there are few clues as to which path or even which general philosophy will prove most profitable.

By contrast, Britain's Alvey Programme, although unashamedly inspired by ICOT's project, now looks quite catholic. It centres on four themes: software engineering, man-machine interfaces, intelligent knowledge-based systems and the development of very large scale integrated circuits, but admits of a variety of approaches. To counter the criticism best expressed by Robert Kowalski, one of PROLOG's inventors, that "it can be argued that it is harmful to support British research, because it is more likely to be exploited abroad than at home", all projects involve industrial participation and (almost) half industrial finance — even university-based projects have to find an industrial "uncle".

This in turn has led to an ICOT criticism of the Alvey project as "too incremental, too much aimed at short-term industrial application and lacking a basic research component" — exactly the complaints that have been flung at Japan's research for the past decade. And the industrial link has smashed hopes of cooperation between the United Kingdom and Japan. ICOT's project is 100 per cent supported by the government and ICOT will hold the patents.

With a budget of £350 million for the five years of the Alvey Programme, expenditure is running at double the rate of ICOT's project. But ICOT is only a tiny part of Japan's advanced computing effort: the telecommunications monopoly NTT's research laboratories have their own fifth generation project, and all of the large electronics manufacturers have artificial intelligence projects.

Only the United States, in total, is exceeding Japan's research efforts. Special projects launched there include the Defense Advanced Research Project Agency (DARPA)'s strategic computing programme (\$600 million over five years), the very high speed integrated circuit programme (\$100 million in 1984), other government programmes and new univer-

sity centres (\$100 million in 1984). There is also the private sector Microelectronics and Computer Technology Corporation and of course AT&T and IBM whose doings remain secret.

The West German response is more recent, stemming from the March 1984 government report on information technology which sets aside DM2,640 million over five years for information technology research. Collaboration between industry and the universities is again specially emphasized: key projects are on computer-aided design, new computer architectures and knowledge engineering.

France does not have a single grand plan, but has ambitions (see page 297). In 1983 the French initiated seven national projects, involving both industrial and public research laboratories, with themes broadly similar to those of the Alvey project. Now in preparation is a much longer-term set of fundamental research projects, centred on the Centre National de la Recherche Scientifique but also involving industry.

Finally, Esprit aims to overcome the European Communities' main weakness — fragmentation of research efforts — and rebuild the research base through "cooperation in precompetitive research and development". In 1984, 110 projects were being supported at 500 companies, institutes and universities, and expenditure of 200 million ECU approved.

All countries report that their biggest problem is finding trained staff — Alvey director Brian Oakley made in plain that anyone studying artificial intelligence will not be joining Britain's unemployed.

Alun Anderson

Apartheid telescoped

A PETITION signed by 156 UK astronomers has been handed to the Science and Engineering Research Council (SERC) calling for its links with the South African Astronomical Observatory (SAAO) to be cut, in the belief "that the continued funding (*sic*) of SAAO by SERC amounts to acquiescence in apartheid". SERC will not respond to the petition because, according to a spokeswoman, such decisions can be made by SERC only on the basis of scientific considerations.

SERC supports SAAO to the tune of about £250,000 a year together with travel and project grants to UK observers, who make considerable use of its facilities. Continuing use of the observatory is to be assessed next month as a matter of routine — though in the context of unusually tight financial constraints — during a "forward look" review by a sub-committee of SERC's Astronomy, Space and Radio Board. The controversy was last aired in 1982 when, as a result of a questionnaire circulated by SERC, it was reported that a majority of the community supported continued use of SAAO (see *Nature* 298, 698; 1982).

Philip Campbell

US research objectives

Keyworth's shopping list

Washington

MORE government support for research and training in biochemical engineering is urgently needed if the United States is to lead commercialization of the new biology, according to advice given by an expert panel to the White House Office of Science and Technology Policy (OSTP).

The panel, convened by the Committee on Science, Engineering and Public Policy (COSEPUP), says the United States lags in basic chemical engineering expertise and that if the position is not corrected, "the Japanese and the Europeans will certainly take the leading role".

The panel concludes that the government has a critical part to play in establishing US expertise in reactor design and purification methods and proposes a variety of grants and awards to be administered by federal agencies. The number of people graduating with higher degrees in biochemical engineering should be doubled or tripled from the present 60 per year.

The research briefing panel on chemical and process engineering for biotechnology is the most outspoken of 9 separate panels organized this year by COSEPUP. The briefings were given to OSTP officials, including director George Keyworth, during the autumn but published only last week.

COSEPUP briefings have over the past 3 years had a significant effect on OSTP policy; the proposed Engineering Research Centers, for example, stem directly from a COSEPUP initiative.

Dr Keyworth has this year taken a more active part in selecting subjects than previously. But while pleas for financial support for particular areas are taken seriously, according to officials, it is thought that some of the matters taken up by this year's briefing panels have already received special attention; in particular, the engineering research centres planned for some university engineering departments may go a long way towards meeting present needs.

A panel on computer architecture has urged immediate efforts to accelerate development of advanced software for the forthcoming generation of parallel processing computers: several indicators suggest that Japanese activities in this field exceed present US capability. And although the United States hopes that it can retain its lead in parallel processing hardware, the panel recommends that a body of specialists should be brought together to follow the Japanese technical literature to warn of any "technological surprises" coming from the much-vaunted fifth generation computer programme. The British Alvey programme of research and development should similarly be "watched closely". But the panel is ap-

parently unimpressed by the potential of the European Esprit programme.

Other areas where COSEPUP panels urge new federal initiatives include advanced polymer composites, laser-atomic interactions and relativistic plasma waves. Research on polymers is badly organized, according to one panel, and there should be a new emphasis on interdisciplinary training to bring together scientific skills needed for development of advanced organic polymers, possibly concentrated at special centres.

Another panel, on physics, argues the likely future importance of accelerators based on electric fields produced in plasma waves, high resolution spectroscopy using relativistic particle beams, and two-dimensional structures. In plasma physics and astrophysics, there is a need for improved computing facilities.

Australian pests

Legal framework for control

Canberra

THE Australian government's introduction of the Biological Control Bill 1984 is another milestone in the long struggle for supremacy over exotic pests. In the meat export trade, a large part of Australia's reputation as a reliable supplier of high-quality produce is the good health of its farm animals, with no outbreak of foot-and-mouth disease since 1872, rabies since 1867 or rinderpest since 1923.

By contrast, the difficulty in enforcing large-scale biological control of weeds and other crop pests in the Australian federal system is nowhere more clearly displayed than in the drawn-out conflict between beekeeper and landowner over the relative merits of the purple-flowered Mediterranean plant *Echium plantagineum* — known as Salvation Jane or Paterson's Curse, depending on one's point of view, and introduced into Australia more than a hundred years ago.

Since the turn of the century, various grazer groups in the sheep and cattle regions of southern Australia have had the vigorous exotic declared a noxious weed, claiming that it detracts from the fodder value of pasture land and produces pyrolyzidine alkaloids poisonous to cattle.

In 1974, all state departments of agriculture responded positively to an inquiry from the Division of Entomology of the Commonwealth Scientific and Industrial Research Organization (CSIRO) to prepare the release of exotic insect agents to control *Echium*. In 1978, supported by graziers from lower rainfall areas, the beekeepers protested that the proposed control programme should not proceed, proclaiming *Echium* the most important honeybee resource growing in south-east

In marked contrast with the recommendations on technology, however, panels reporting on opportunities in basic biology appear content with the existing federal role. An overview of the biology of oncogenes produced by a panel under the chairmanship of Dr Robert Weinberg of Massachusetts Institute of Technology, for example, concludes that further progress towards an understanding of cancer "will depend on continuing support of the basic biologic disciplines whose successes have proved so useful...".

A report on interactions of blood and blood vessels stresses the potential health benefits to be gained and advocates more research but demands no government action. The contrast has not been lost on OSTP. Parasitism, however, is identified as ripe for a major expansion, as molecular biology provides new techniques. Here, as in other areas, OSTP is told of a need to encourage more scientists to study the basic biology of the organisms involved.

Tim Beardsley

Australia and in some years the only spring crop available.

The controversy seesawed for two years until, in 1980, the *Echium* leaf mining moth *Dialectica scallariella* was released at three experimental sites in New South Wales. But later that year, the high court granted a restraining injunction on narrow grounds in favour of beekeeper and low-rainfall-zone grazier plaintiffs. CSIRO was required to halt importation of further European insects and to destroy those already released. Another agreement was reached in June 1982 to set up a tribunal to determine whether control of *Echium* should proceed at all, but this did not materialize.

Throughout the whole litigation period, which ended in June 1983 with CSIRO agreeing to a permanent injunction, the federal government had only a limited capacity to enact far-reaching biological control laws. Except for quarantine, most constitutional power in the area of exotic disease control, prevention and compensation resides with the states.

In August this year, however, the government introduced the Biological Control Bill which has passed in both houses and will soon become law, but only in the Australian Capital Territory. It provides for widespread advertising of proposed control programmes and a commission of inquiry and powers of review for individual cases. The bill in effect negates liability for the approved release of control organisms and is meant as a model for complementary legislation in the states. But because of the inability of either pest or bug to discern state boundaries, opponents of the bill will have to lobby successfully in only one state to disable the entire scheme.

Jeffrey Sellar

European computing

French bid to build centre

TOULOUSE in the south of France — a French centre for the aerospace industries, biotechnology and astrophysics — is making a serious play to become a major European focus for research and training in advanced scientific computation. The aim is to bring together computer scientists interested in new architectures (array or parallel processing, multi-instruction-stream computers and beyond), users and numerical analysts in a melting-pot that would be unique at least in Europe.

The Toulouse centre as at present planned would at least match a combination of the US National Aeronautics and Space Administration (NASA)'s Langley Institute of Computer Applications in Science and Engineering (ICASE) at Hampton, Virginia and the new NASA Research Institute for Advanced Computer Science (RIACS), which started just a few months ago at Ames, California.

This is not, however, a European "fifth generation" artificial intelligence (AI) experiment, although the Toulouse association for the promotion of the Centre Européen de Recherche et de Formation Avancé en Calcul Scientifique (CERFACS) wants to have available the most advanced computer possible, and is aiming to have its real impact in the 1990s.

The CERFACS objective is less ambitious than AI, and possibly more realistic. It stems from a growing feeling among "number crunchers" of all kinds that the new computer architectures and components require radically new techniques, and that interaction between users and computer designers is becoming essential. For example, says Professor Roger Hackney of the Department of Computer Science at the University of Reading, architectures designed to match particular problems can now be "a very cost-effective way of getting large computer power to the problem".

The best use of future and existing machines can be best learned and developed, it is felt, in a centre combining the disciplines of computer science (architectures), numerical analysis (algorithms) and applications specialisms. CERFACS would be such a centre. Nothing like it exists in Europe, Hackney claims. Moreover, NASA's ICASE and RIACS, while addressing all the relevant issues, concentrate architecture on the west coast and applications on the east.

Training and networking would also be "very important" at CERFACS. Visitors would come for several weeks or months, but then return to their home institutions, from which they would wish to keep in touch with CERFACS and use its computing power. So a satellite-based computer communications network is also proposed.

But will CERFACS get off the ground? The idea was the brainchild of Jean-Paul

Zahn, director of the Pic du Midi Observatory near Toulouse, but it has now been taken up with enormous enthusiasm by local industry and politicians such as the local mayor and European parliamentarian Henri Sabi.

However, the news from Brussels is not bright. Although the European Commission has set up a "study" of CERFACS, "we learned last week there's little prospect of Brussels cash in the next five years", said a CERFACS spokeswoman in Toulouse last week. "It just takes that long for the Commission to set up a programme."

So the hunt is on for support from the French government (said to be "very positive" to the idea), and from elsewhere. A European support committee has been established with members from the United

Kingdom, West Germany, the Netherlands, Spain, Greece, Denmark and Switzerland, which will report back early next year with the results of its approaches to government and other agencies.

CERFACS does have one guarantee: a purpose-built building. The Midi-Pyrénées regional government will pay for that. Internationally, Greece has already shown great interest, and smaller European nations may well prove more favourable than, say, Britain and West Germany, which already have large machines — even if they do not use them in the CERFACS manner.

France, however, is well behind Britain and West Germany in scientific computing power, so a conceivable outcome is that France will set up a joint arrangement for CERFACS between itself and the smaller nations of Europe. Whatever emerges from present soundings, a formal proposal to the French government will follow, probably in February. **Robert Walgate**

NIH

Academy recommends guide

Washington

A NATIONAL Academy of Sciences panel, after a year-long study, has suggested that the National Institutes of Health (NIH) might best defend themselves against the pressures of the "disease of the month club" by the time-honoured government strategy of injecting a new layer of bureaucracy into the organization. In recent years, a growing number of disease lobbies, acting through Congress, have pressed for the creation of new NIH institutes bearing the name of "their" disease. NIH has resisted these forces with varying success, but recently scored a victory when President Reagan vetoed a bill that would have established new National Institutes of Arthritis and of Nursing.

The academy recommendation is that there should be a new advisory body, to be called the Health Science Board, whose major purpose, it appears, would be to rechannel the public pressures that now find expression through Congress. The board would "communicate public perceptions of health research needs" to NIH and other Public Health Service agencies and would "assure the public that these needs are being . . . addressed".

Proposals for new NIH institutes would be subject to a formal review by the board, which would apply five criteria that would specifically exclude institutes whose major function is regulation, health services or other non-research activities. By these criteria, the National Institute for Occupational Safety and Health and the National Centers for Health Services Research and for Health Statistics — all of which, at some time, have been considered for incorporation into NIH — would be excluded.

The academy panel would not comment on specific proposals, such as that for an

arthritis institute, but advised that with NIH now having grown to 11 institutes, there should be a "presumption" against new additions. The panel's survey of NIH's organizational and budget history failed to produce evidence to substantiate the most often-heard justification for new institutes — that they boost support for the particular area of research and thus for NIH as a whole. (A spokesman for the Arthritis Foundation, for example, told the panel at a public hearing last year that by so visibly responding to such politically-popular health issues as arthritis, NIH could only gain. "We must see how we can take advantage of the system.")

By contrast, the panel concluded that new institutes add overhead costs, add to the administrative burden of an already overburdened NIH director (25 officials already report directly to him) and hinder scientific communication within NIH.

If the assurances of the Health Science Board are not enough to convince the public that their perceived health research needs are being met, NIH could avail itself of a "continuum" of options short of creating new institutes, the academy panel said. These options include "publicizing what scientific research has accomplished", holding conferences, naming special panels and creating new divisions or programmes within existing institutes.

A somewhat more tangible recommendation of the panel in helping NIH to respond to changing health research needs is for the creation of a contingency fund of 1 per cent of NIH's budget that the director could apply to health emergencies. At current spending, that one per cent equals about \$50 million, which may be more of a slush fund than Congress would be willing to create. **Stephen Budiansky**

Whaling

US and Japan agree to disagree

Tokyo

ANGRY criticism from press and politicians has greeted the Japanese government's decision to bow to US pressure and stop all sperm whaling by 1988. But there is some confusion over exactly what Japan has agreed to, with conflicting announcements in Washington and Tokyo, and protests from Greenpeace (the environmental pressure group) that Japan has got off far too lightly.

According to US negotiators, Japan has agreed to end by 1988 not just sperm whaling but all whaling. With this agreement, the United States hopes to avoid the bad feelings that would arise if, instead, it tried to use cuts in fishing quotas to force Japan

IWC ruling is not diminished.

Japan is, in any case, far more concerned about minke whales than sperm whales. At the same IWC 1984 meeting where the sperm whale quota was cut to zero, the Antarctic minke whale quota — upon which Japan's offshore whaling industry depends — was cut to 4,224 to be shared between Japan, the Soviet Union and Brazil. Both the Soviet Union and Brazil have formally objected to the quota, thus freeing themselves from obligation to abide by it. Japan has not so far objected because of its fear that US fishery cuts would follow. But immediately after the new agreement with the United States was announced, the agriculture, forestry and fisheries minister Moriyoshi Sato stated

that Japan will file an objection to the minke whale quota before the deadline of 6 January. The following year, the minke whale quota would automatically fall to zero as the total ban on whaling agreed by IWC in 1982 comes into effect.

It seems clear that the minke whale quota has been the subject of a US offer that goes over IWC's head: a blind eye would be turned to further whaling in the next three years and no fishing quota cuts would be imposed, provided Japan will compromise and agree to stop all whaling by 1988. Washington has apparently gone so far as to claim that Japan has already agreed to accept this offer. The subsequent announcement from Mr Sato suggests, however, that Japan will still fight on and try to win some further concessions from the United States — if legal action does not in the meantime undo what has already been achieved.

Alun Anderson



to abide by the rulings of the International Whaling Commission (IWC) that sperm whaling should end this year, and all commercial whaling next year. But Japanese officials claim they have agreed to end only sperm whaling by 1988 and that a ban on whaling altogether is only a US offer. And an angry statement from Hiroya Sanyo, the Fisheries Agency director general who headed the Japanese delegation, adds that the talks had been "unfair" and "outside the bounds of normal diplomacy".

Anti-whaling groups in Western nations are equally angry that Japan is now to be given until 1988 to end sperm whaling. An injunction is to be sought to force the US government to obey the Packwood-Magnusson Amendment to the 1979 Fishery Conservation and Management Act and impose cuts in Japan's fishing quotas the moment Japanese boats catch a sperm whale — and there are unconfirmed reports that two whales have already been caught.

The Packwood-Magnusson Amendment provides that when a nation "diminishes the effectiveness" of an international whaling agreement, it must be "certified" by the US government, which means that that nation's quota in the US 200-mile zone must be cut by at least half in the first year of certification and by 100 per cent in the second year. The outcome of court action is now likely to hinge on the meaning of the words "diminish the effectiveness", with the US government arguing that with the new agreement the effectiveness of the

Around the world in four years

OPERATION Raleigh, a four-year circumnavigation of the world that plans to mount over 150 scientific projects in more than 30 countries, was launched last week by its British patron, the Prince of Wales. The flagship of the expedition, named for Sir Walter Raleigh who sponsored the first English-speaking colony in North America 400 years ago, sailed from Kingston upon Hull last week for the Bahamas, on the first of 16 three-month legs.

The expedition is modelled on Operation Drake (1978-80) and is organized by the Scientific Exploration Society Ltd, a registered charity. Operation Raleigh will involve over 300 scientists, assisted by 4,000 selected young people, 75 per cent of whom will come from the United States and Britain. The ship is equipped with three scientific laboratories largely donated by Gallenkamp, diving facilities including a decompression chamber, a Marisat satellite communications system and a computer centre donated by Acorn Computers Ltd and Centronics, and will support both marine and land-based projects.

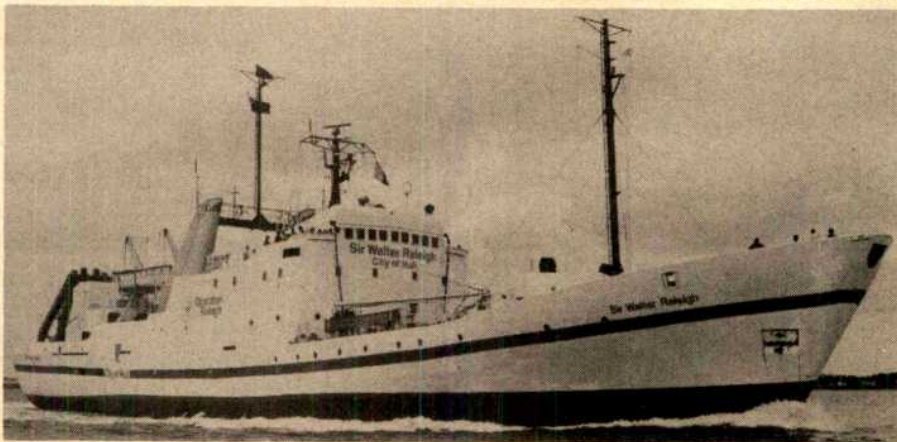
The first leg of the expedition to the Bahamas and Turks and Caicos Islands in the West Indies will include a biological

and geological investigation of the "blue holes" system of the Bahamian islands — underwater caves and tunnels carved out by the dissolution of limestone rocks by fresh water at a time (about 18,000 years ago) when the sea level was about 100 metres lower than it is now.

The Bahamas will also be the first site in a "reefwatch" programme to monitor the effects of sediment pollution on coral reefs. Coastal accumulation of sediment resulting from forest clearance reduces the amount of light penetrating the coral, which has an adverse effect on the photosynthesis of the symbiotic algae on which reef-building corals depend for their survival.

Operation Raleigh, operating as it does mainly from tropical latitudes, might be considered a "jamboree" for the scientists involved, but the main criterion used by the Scientific Research Committee in selecting projects — which must be more or less self-financing — was that the work should produce results suitable for publication in research journals. The young people who will provide semi-skilled assistance on the expedition were also subject to rigorous selection procedures.

Peta Pickering



Vaccinia virus

WHO to back vector vaccines

Washington

A SPECIAL advisory group to the World Health Organization (WHO) has approved an accelerated programme of research on recombinant vaccines using viral vectors. Such vaccines, now under development, might be used in mass immunization programmes where alternatives do not exist or are prohibitively expensive. Though yet to be approved by WHO's regular scientific advisory group, the decision puts to rest speculation that the reintroduction of vaccinia immunization programmes might be thought unnecessarily dangerous.

Vaccinia (cowpox virus) has long been a favourite organism for virologists, being easy to culture and safe to handle. With the elimination of smallpox, its use in vaccination was discontinued, because of severe reactions, sometimes fatal, that occur at the rate of 1 or 2 per million vaccinations. Furthermore, vaccinia can be transmitted by skin contact between people and from animals, albeit rarely.

Despite some reservations expressed at a workshop held last week jointly by WHO, the US Public Health Service and the British National Institute of Biological Standards and Control, the advisory group decided that the potential benefits of research into recombinant vaccines outweigh

the possible risk. Protection against a number of diseases has already been demonstrated in animals using vaccinia as a vector for antigens, including hepatitis B, herpes simplex virus type 1 and rabies (see for example *Nature* 311, 67; 1984). Although the safety of these vaccines has not been systematically studied, it appears that deletion of the thymidine kinase gene of vaccinia — which is replaced by the foreign gene — may make the virus even less easily transmitted than the normal type.

In its advice to WHO, the advisory group stresses the importance of developing alternative vectors likely to be of use against diseases of the respiratory tract and enteric diseases, including bacterial, protozoal and helminthic diseases. Because much early experience will be with animal vaccines, WHO is urged to establish links with the United Nations Food and Agriculture Organization to monitor veterinary results. And to avoid countries using different strains of vaccines haphazardly, WHO is invited to set up a collaborative centre for dissemination of information and storage of reference samples, probably to be located at the National Institute for Allergy and Infectious diseases at Bethesda, Maryland. **Tim Beardsley**

Israeli education

Palestinians push ahead

Rehovot

THE latest of Israel's institutions of higher learning, Ben-Gurion University of the Negev, has finally begun its academic year, two weeks behind schedule. It remains to be seen, however, how long Ben-Gurion and the others — all plagued by severe financial problems — will remain open.

To be sure, Ben-Gurion University, with an accumulated debt of US \$15 million, is worse off than the older, better established institutions. And this presumably explains both the delayed opening and the resignation (later withdrawn) of university president Shlomo Gazit. But with government support for higher education having dropped by one-third in recent years, the other schools are not in much better shape. The president of the Haifa Technion, Professor Josef Singer, has stated that "the Technion is still \$10 million short of even its reduced emergency budget and the conditions for an uninterrupted school year have not yet been guaranteed".

There is greater sympathy for the plight of the universities among members of the present government than existed in the last one, and education minister Yitzhak Navon, for one, has described their situation as "intolerable". But with all public spending being drastically reduced, including substantial cutbacks in even

defence and social welfare, it is unlikely that the government will be more generous than it has been in the past: in all probability, support will decline still further. This has forced the universities not only to undertake large-scale dismissals and reduce their purchases of vital scientific equipment, but also to shut down whole departments.

This development is taking place against the background of a dramatic increase in the educational level of Palestinians, both in Jordan and in the West Bank. During the 1983-84 academic year, 25,000 Jordanians and 13,000 West Bank Arabs passed the Jordanian matriculation examinations, about twice the number of matriculants as among the Jews of Israel, from roughly the same population of some four million people.

Educational advancement has been particularly spectacular in the territories under Israeli control since 1967. From 1967 to 1982, the secondary school population in the West Bank and the Gaza Strip increased by 106 per cent, while the general population went up only 23 per cent; and while in 1967 there were just a few hundred students in post-secondary programmes in those two areas, this year there will be nearly 20,000, a very high proportion.

Nechemia Meyers

Gromyko's prize

THIS year's Soviet State Prizes for science, announced as usual to coincide with the anniversary of the 1917 October Revolution — in November — went almost entirely to "cycles of works" carried out by teams of up to 12 persons over a long period of time. Only four prizes of the fourteen went to monographs by a single author: Ilya N. Vekua's "Some general methods of constructing different variants of the theory of shells" (1982), Dr Andrei D. Ado's "General allergology" (1978), Dr Valentin V. Seldov's "The East Slavs in the sixth to thirteenth centuries" (1982) and foreign minister Andrei A. Gromyko's "External expansion of capital: past and present" (1982).

Several of the "cycles" honoured cover two decades or more including B.B. Bokut' *et al.*'s "Highly effective nonlinear transformations of frequency in crystals and the creation of returnable sources of coherent optical radiation" (1963-82), I.N. Matrosov *et al.*'s "Elaboration of the method of Lyapunov vector functions for the analysis of stability and other dynamic properties of nonlinear systems" (1962-81) and K. P. Belov *et al.* "Magnetism and the electronic structure of rare-earth and uranium compounds" (1959-82). One citation seems to have been overdue — that of N. N. Bogolyubov, D. V. Shirkov and A. A. Logunov for their contribution "Method of renormalized groups in field theory", which carries the date 1955-56.

The citations do not make it clear just how far a cycle of work consists of genuine collaborative undertakings and how far it relates to parallel but separate work on a common theme. Co-winners are frequently scattered throughout several institutes, and even republics. Thus Bokut' *et al.* come from Gomel' University (Byelorussia), the Vavilov State Optical Institute, Moscow State University, the Institute of General Physics of the Soviet Academy of Sciences, Vilnius State University (Lithuania), the Institute of Electronics of the Uzbek Academy of Sciences and the Institute of Applied Physics of the Soviet Academy of Sciences. On the other hand, the team of geologists honoured for their complex study of the Baltic regions of the Soviet Union were all, to judge from their names, native to the Baltic republics (Latvia, Lithuania and Estonia) and for the most part are employed in research institutions of those republics, although one holds an all-Union post, as deputy director of the Marine Scientific Research Association of Engineering Geology. Likewise the team responsible for "Development of the theoretical principles of Soviet criminology" (1961-82) — a unusual subject for a state prize — all seem to be Moscow-based, although affiliated to such diverse institutions as the Academy's Institute of State and Law and the Moscow Police College. **Vera Rich**

United States in UNESCO

Withdrawal sanctioned by default

Washington

AN independent panel appointed to monitor the activities of the United Nations Educational, Social and Cultural Organization (UNESCO) has failed to find persuasive reasons why the United States should reverse its decision to withdraw from the organization at the end of this year. A final decision on US participation in 1985 must be made within the next two weeks, and barring an unexpected change of heart by the State Department, the withdrawal will go ahead as announced at the end of 1983.

The US decision was made because of management inefficiency at UNESCO and "politicization" of its programmes. The monitoring panel, chaired by Dr James Holderman, chairman of the US National Commission on UNESCO and president of the University of South Carolina, makes no specific recommendation on the question of withdrawal. However, its conclusions are thought not to be so compelling as to demand the positive action required to abort withdrawal at this late stage.

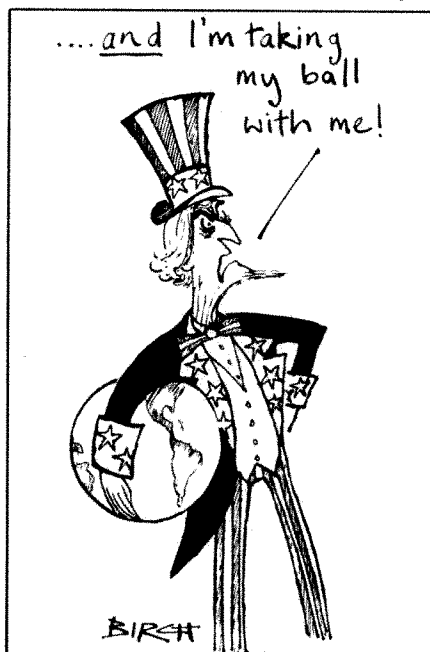
UNESCO has made a number of changes over the past year that go some way to answering the US criticisms. The most important, from the US point of view, was the recent agreement to freeze the 1986-87 budget at the 1984-85 level, a move long demanded by the Reagan administration. Furthermore, some of the more ideologically inspired language used to describe UNESCO ambitions on disarmament and human rights has been toned down, and new efforts have been made to evaluate programmes, both actions that indicate some sensitivity to US concern. But recently agreed management reforms are not thought by the United States to go far enough and there is considerable scepticism about how rapidly and effectively the agreed changes will be implemented.

Despite the partial successes, however, the United States has failed totally to bring about changes in the voting structure at UNESCO, and was deserted by its Western allies on this issue. As in other United Nations organizations, there is one vote per country, even though financial contributions are in proportion to countries' gross national products. The US contribution, at \$47 million, amounts to one quarter of UNESCO's total budget. Although UNESCO officials express confidence that programmes will continue even if the withdrawal does go ahead, the impact will be serious — especially if other countries follow the US lead. Britain and West Germany are wavering.

A separate assessment of how UNESCO has accommodated US demands is being undertaken by two subcommittees of the House of Representatives' Foreign Affairs

Committee, under Representatives Gus Yatron and Dan Mica. This assessment will not however be complete before February, and so will not bear on the withdrawal decision. In any event, the executive decision to withdraw would not easily be swayed by a congressional report. At one stage, the possibility was considered that the United States might delay its withdrawal by one year, which would bring it neatly to the end of a UNESCO biennial programme cycle, but that option has now been definitely discounted by Gregory Newell, Assistant Secretary for international organization affairs at the State Department.

The US decision had dismayed many who were working on scientific projects administered by UNESCO, which were thought to be largely innocent of the faults levelled at other programmes. The National Academy of Sciences earlier this year



produced a favourable assessment of the scientific benefits obtained through the agency. But the State Department maintains that in most instances US scientists will be able to continue their involvement with UNESCO programmes even after withdrawal, despite the lack of US representation at council level: domestic expenditure on the Man and the Biosphere programme, for example, is scheduled to be doubled or tripled over the next few years.

The State Department says it is also looking for ways to encourage technological development in developing countries that do not rely on UNESCO. How far these aims prove possible will depend on the seriousness they are accorded by the Office of Management and Budget, and on the willingness of UNESCO to cooperate after being jilted by its biggest paymaster.

Tim Beardsley

West German forests

Damage survey looks bad

Munich

THIS year's inventory of damage to West German forests, the first results of which were published earlier this month, shows further deterioration since last year. A half of all forested areas are now believed to be affected to some degree, compared with 34 per cent in 1983. But as yet there are no reports of forest areas in which the damage is complete.

The Forest Damage Inventory now completed was initiated by the federal government in Bonn, and is the third of a series begun in 1982. For the first time, the survey was based on a nationwide system of 4 km × 14 km plots. This year, however, the classification was refined so as to include five categories of damage (rather than three), numbered 0 (undamaged) to 3 (severely damaged) and 4 (dead).

The main criterion for assessing damage is the extent of loss of needles or leaves, with the discoloration of foliage a secondary consideration. The survey earlier this year was carried out by specially trained forestry personnel carrying colour photographs with which to check their observations.

Between 1983 and 1984, the most obvious change has been the increase of the proportion of West German forests in which the damage is classified as slight or moderately severe. Between the two most recent surveys there has also been a dramatic increase in the extent of damage in some degree to broad-leaved trees, with the percentage of affected beech trees increasing from 26 to 50 per cent between 1983 and 1984, and that of oak trees from 15 to 43 per cent.

In the latest survey, damage has been reported from throughout West Germany, but is most serious in the southern *Länder* of Bavaria and Baden-Wurtemberg. While the earlier surveys showed damage predominantly at high altitudes, the most recent survey shows damage everywhere.

As in previous years, the results of the forest inventory will not go undisputed. Although the methods used in 1984 have been more systematically applied throughout West Germany, the validity of needle loss as the main criterion for assessing damage is likely to remain controversial. But whatever is happening, there can be no dispute that the new type forest decline is increasing both in geographical extent and in severity.

Although the cause of the damage remains open to debate, in the past few years increasing attention has been paid to the importance of ozone in conjunction with other stressing factors as the chief agent of damage. So far, there have been no reports of forest tracts in which all trees have been killed.

L.W.Blank

Conditions for disarmament

SIR — J.N. Barkenbus and A.M. Weinberg (*Nature* 310, 95; 1984) were right to object that Stephen Salter's proposal for a superpowers disarmament negotiations algorithm (308, 490; 1984) assumes an unrealistic degree of collaboration between the protagonists. But it may slightly misleading to term the Salter scheme "original". Undoubtedly it is Mr Salter's own autonomous creation, and in its particular form it may also be entirely novel. It is certainly both elegant and economically structured. However, the "I cut — you choose" algorithm was suggested for this purpose in an article in *Journal of Conflict Resolution* 1963 by E. Singer.

The many differences between the Singer and Salter versions of what the former terms the "divide and choose" approach are probably of little interest, except to those who set more store by the contemplation of abstract negotiation models than I do. Suffice it to notice here that Singer's approach was to place all weapon retentions and weapon discards by both sides into a single population to be divided, and then to consider solutions to the difficulties this could pose. He also throws out the interesting suggestion that on-site inspection rights could be bargained for in the same or another similar structure, after they had first been portioned out in territorial units.

Unlike Salter, Singer did not so much see his scheme as replacing the antagonistic conduct of international relations, but rather as having to take its chances within the sordid arena of diplomacy. Thus he concludes: "Eventually, the realities of international negotiations must be confronted, and the diplomatic foxes must be set free to see if they can, without too much difficulty, play havoc with a bargaining model".

It is a pity that Barkenbus and Weinberg were unable to espouse the realism whose absence they regret in Salter. For it is certainly very naive to suppose, as their model does, that the arms race is about preserving a stable parity between the deliverable portions of populations of militarily undifferentiated "warheads". Their second and probably related assumption, also hard to share, is that we are dealing always and only with retaliatory forces. For once the idea of an attempted disarming first strike is admitted into the strategic calculations (as it always has been on both sides), it is no longer possible to claim that erection of a defensive anti-missile system can be directly offset by dismantling a proportion of one's own offensive missiles, to match the proportion of an enemy missile strike one expects to be able to block. For suppose Side A, with its new defensive system and, say, a matching 10 per cent reduction of offensive forces, were now to initiate a strategic first strike. The 90 per cent offensive force would be virtually as effective as before the reduction, and the defensive force,

facing only the decimated and disrupted remains of B's strategic offensive capability, would be very much more than 10 per cent effective because it would be handling a much smaller salvo of incoming warheads.

Since this is the worst-case scenario which would govern B's perceptions of A's new defensive deployment, it could be argued that the latter should be offset by a reduction of A's offensive arsenal proportional to the hypothetical "thinning" by which B's offensive forces would receive by the two processes of, first, an attempted disarming first strike by A, and second, the anti-missile effects of A's defences on B's subsequent retaliation. Thus A would have to pay for its 10 per cent effective defences with, say, an 80 per cent reduction of its offensive forces.

But of course A could not accept this, because it has to see the world as one in which B could be making the first strike, which demonstrates that there is no way to provide a "fair" mix of offensive and defensive deployments, except by making artificial and unrealistic assumptions to the effect that all nuclear strikes will always be retaliations, which is of course a useless option, despite Barkenbus and Weinberg's so boldly having taken it. R.I.P. BULKELEY
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SIR — The leading article "Targets for future arms control" (18 October, p.591) was both pessimistic and realistic. In a climate of mutual and reciprocal suspicion between the nuclear armed states, perhaps we should look to small, but achievable, measures that both build confidence and reduce further escalation of the nuclear arms race. I propose a measure that might achieve this and at the same time bolster the Non-Proliferation Treaty (NPT), which, you rightly comment, mercifully still holds, but only tenuously.

With 50,000 or so nuclear warheads worldwide, there is surely no need to add to this total. If so, then an effective "freeze" on fissile material production should be agreed to and implemented by each of the nuclear weapon states. A partial freeze of this sort was agreed by the US, UK and Soviet governments in April 1964.

In order to implement this, the nuclear weapon states would have to open up their reprocessing plants and plutonium/enriched uranium stockpiles to full inspection by a supra-national agency.

In his book *Face the Future* (1981, pp. 488-89), former British Foreign Secretary Dr David Owen has already argued this, basing his conclusions on his experience in office. With the Soviet Union recently accepting International Atomic Energy Agency (IAEA) safeguarding on some of its civil nuclear facilities and with the People's Republic of China joining IAEA,

the foundations of progress exist.

The French and British dual-purpose nuclear facilities, such as Marcoule and Phénix, and Sellafield remain a problem. Indeed Dr D. G. Avery, appearing on behalf of British Nuclear Fuels PLC, told the Sizewell B Inquiry (16 October) that the British Government and Euratom have been at loggerheads since 1973 over the non-accessibility of the Sellafield reprocessing line to physical inspection, despite Britain's accession to the Euratom treaty as a full partner.

Nonetheless, this problem certainly seems surmountable. This being so, it would surely strengthen the NPT, whose review conference in September 1985 is surely going to demand more equitable behaviour by the nuclear weapon states if others are not to withdraw, with the disastrous consequence of a nuclear free-for-all. DAVID LOWRY

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Family planning

SIR — The reduction of the birth rate in China by one-half during the 1970s, reported in *Nature* (310, 612; 1984), is indeed welcome news. In view of high levels of fertility persisting in many areas of the world¹, what are the prospects for this feat to be duplicated elsewhere?

The primary requirement for a successful population programme must surely be the participation of most of the population in the programme; yet even in campaigns as vigorous as that in India, fewer than 1 in 4 eligible couples are protected². So the choice of method may be of secondary importance — pills and rhythm, for example, seem to have a similar overall impact on the fertility of acceptors³.

How can the scope of population programmes be broadened in societies which are less highly centralized than China's? A mass-marketing approach may be effective, as demonstrated by the unfortunate popularity of artificial infant feeding formulas in even the poorest areas. A more beneficial twist of this technique might involve the use of some other highly marketable health product in conjunction with a family planning campaign. Due to the threat of anaemia (see for example *Nature* 310, 615; 1984), iron supplements would be beneficial and would have a high level of appeal to women. By distributing supplements in packets of 28 and colour-coding them for the safe and unsafe days of the menstrual cycle, for example, many more women might be introduced to the practice of family planning than have been reached by conventional programmes.

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Moral responsibility

SIR — In the leading article (*Nature* 25 October, p.692) on the expulsion of the ex-Nazi scientist, Rudolph, from the United States, the statement is made that “scientific research is a neutral, amoral enterprise *only* in a free society, one with a democracy capable of exercising social judgement over the propriety of end uses”. I would contend that you let us scientists off too easy, that we all have a moral responsibility, maybe even a duty, not to work on obviously immoral projects, such as, for example, those involving the murder of innocent people by chemical, biological, atomic-nuclear and even “conventional” weapons. Indeed, in the present international situation where warfare is not likely to be between individual soldiers but between peoples of contending states, it is the moral responsibility of scientists everywhere not to work on any project connected with waging war. I know the answer to this: that we in the democratic societies leave ourselves open to “nuclear blackmail”. But are you so sure of that, as much as you seem to be unsure of the great possibility that the ludicrous, to say the least, build-up of weaponry will not lead to a nuclear-driven mass destruction?

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Red Sirius

SIR — In the light of the discussions in *Nature* of Homer's wine-dark sea during the past four months, it was almost inevitable that the old problem of the red colour of Sirius would arise sooner or later (*Nature* 6 September, p.8).

However, the problem is soluble, from the testimony of several independent observers.

Arguing for a red colour of Sirius, only two referees may be regarded as reliable: the Roman philosopher Seneca (~70BC)¹ and the Alexandrine astronomer/astrologer Ptolemy (~AD150)². Neither texts, however, gives particulars of how these observations were made. All other Greek and Roman texts often presented as additional evidence³, speak only of the “fires” and “flames” of Sirius as it scorches the Earth during the “Dog-days”.

An Assyrian text (BM 118898)⁴, dating from the reign of Ashur-bel-kala (~1070BC), mentions a colour “red as molten copper”, but here Sirius is clearly stated to be rising and was therefore observed close to the horizon. The same applies to the observations described by the Arab poet Ibn Alrakka (as preserved by al-Biruni (1000)⁵).

Refuting such evidence are a number of contemporary texts citing a white or blue colour for Sirius. Astrological texts are very useful as stellar and planetary colours were closely observed for inferring future

events. The Greek astrologer Hephaestion Thebanus (~AD400)⁶, citing older Egyptian sources (2BC), discusses the various prognostications derived from the observed colours (golden, red, green and white) of Sirius at its heliacal rising. These colours clearly result from the effects of atmospheric extinction and scintillation on a white star near the horizon. Also, the Roman astrologer Manilius (~AD10)⁷ refers to the cold sea-blue quality of the rays of Sirius.

Most explicit are the observations found in the astronomical chapters of the ancient Chinese dynastical histories. Sima Qian (~90BC)⁸, Ma Xu (~AD100)⁹ and Li Shunfeng (~AD635)¹⁰, discussing the colours of the planets and selected standard stars, all assign to Sirius a white colour.

This evidence clearly shows that Sirius has never been intrinsically red during the past few millennia and is in full accord with the present evolutionary phase of the Sirius-system.

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Italian slur rebutted

SIR — I was pleased to read in *Nature* (11 October, p.501) a news item wishing well to the new president of the Italian National Research Council (CNR). Indeed, Luigi Rossi Bernardi is a scientist first and a science administrator second, and his previous record is one of achievement through hard work, determination to assess quality and ability to develop rational policies. It is on these objective grounds that his appointment deserves praise. The sensitive reader will perceive this from the report. But it is unfortunate that in his opening paragraph Robert Walgate gives the impression that a major implication of the appointment is a transition from the “underdeveloped south” to the “better organized north”. To this the sensitive reader may react emotionally. However, since *Nature* is a

scientific journal, let us look at the issue scientifically.

In volumes 301–306 (1983) of your journal, there are eight papers from Italian institutions. Of these two are from the south, four from central Italy, one from the north. The eighth paper, interestingly, was a collaboration among laboratories in the south, centre and north (Catania, Perugia, Torino). Such a small sample has no claim to statistical significance, but it does not support the notion of a north-south dichotomy with respect to research output, which is what CNR is about. More important, if we look for example to modern biology, it is quite obvious that the main thrust over the past twenty years has come from laboratories in Naples, which, together with other CNR institutes that have developed from them, have today the highest concentration in the country of research and training programmes in molecular genetics and molecular biology.

Nobody will deny the existence of a north-south gradient in economic development in Italy, the historical reasons for which are complex and defy any facile analysis. But it does not become *Nature* to perpetuate ill-founded clichés. The first medical school in the Western world was established in Salerno, near Naples, more than a thousand years ago. Today, among the hardest-working scientists some are Neapolitans working in Naples. One of the challenges facing the new president of CNR is to provide the physical facilities required for a more productive outcome of their efforts and of the talent of their young students. I am convinced that Luigi Rossi Bernardi will be equal to this challenge.

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SIR — It is unpleasant to read in a scientific journal such as *Nature* an article carrying racist and superficial judgements about Italy and the state of its science (“New Hope for Research Council” *Nature* 11 October, p.501).

Phrases such as “colourful Neapolitan presidency” or “underdeveloped south of Italy” betray profound arrogance and ignorance. You also say that the new CNR president “as a scientist” promises a scientific management of the research council that clashes with his political friendships (a truly scientific asset) and the most incredible statement of all — “. . . he comes from a medical department, medics being ‘the strongest mafia — in the best sense [my italics] in the Italian universities’”. I agree on the strength of that particular mafia. But I am sceptical of any “best sense” underlying that lobby. Mafia is mafia and no benefit can come from it to Italian science.

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Genetic engineering and pharmaceuticals

from John Vane and Pedro Cuatrecasas

Genetic engineering now allows biological synthesis and large-scale production of several proteins with therapeutic potential. The principal challenge in this sphere is to identify new, medically and commercially significant targets — the province of cell biologists, physiologists and biochemists. In the future, genetic engineering will surely provide invaluable tools for the study of the molecular basis of cellular control and pathophysiology, which will permit biochemists and medicinal chemists to design novel medicines.

THE essential ingredients of profitable new ethical medicines are chemicals, which in the past have been extracted from plants (digitalis), animals (insulin, vaccines), living cell cultures (vaccines, interferon) or prepared by the synthetic chemist (most new drugs this century). What lies in the future with the advent of genetic engineering?

Medicines from organisms

The extraction of chemicals from organisms has been a source of important therapies for hundreds of years, giving us some of the most fundamental of today's medicines, including digitalis, curare, ephedrine and atropine. Extraction of the ergot fungus growing on rye led to Henry Dale's discoveries and investigations of acetylcholine, histamine and adrenaline. More recently, the extraction of moulds, initiated by the penicillin saga, has culminated in a whole series of antibiotics, the -mycins and the -sporins, for example. Very recently, avermectins have been discovered in extracts from micro-organisms.

The surface of the Earth continues to be combed for further novel sources of therapeutic agents, although such efforts have become less productive in recent years. In the past 15 years, the search has even turned to the bed of the sea, but the Roche experimental marine station, set up in Australia for that purpose, has now been closed. The US National Cancer Institute's search for natural products led to the discovery of potent plant alkaloids but the programme has been curtailed. Other interesting leads may arise from recent studies of Chinese medicinal preparations. But we may have to accept that the therapeutic natural products have largely been discovered.

How, if that should be the case, shall we maintain the therapies the natural products have provided? Digitalis, the cardiotonic drug, used in medicine in one form or another for at least 200 years and which stimulates the failing heart to beat better, is a good example. The "digoxin" of the 1990s will come from one or more of the following sources:

- The ground-up leaf of the foxglove, as at present.
- The leaves of "super-foxgloves" made to yield more digoxin than before, either by standard plant-breeding techniques or by genetic manipulation.

- A tissue culture technique, based on growing foxglove cells as a slurry or on using immobilized plant cells.
- Chemical synthesis of the complex active chemical in the laboratory and factory, provided that product is cheaper than the three above.
- Replacement of digoxin by a therapeutically superior simpler synthetic chemical that does a similar job, as evidenced by animal and clinical experiments.
- The discovery of an endogenous digoxin-like substance that we all make when healthy, but which is deficient in disease. It is possible that such a substance exists (the natriuretic hormone)¹ and that digoxin stimulates the sites on cells designed to react to this natural substance. The identification of such a substance would provide a different starting point for the synthetic chemist.

Digoxin is not a protein, and the genetic information required for its synthesis in the foxglove is probably so complicated that it is unlikely, in the foreseeable future, that gene transplants into other organisms will lead to cheaper synthesis. Thus, for digoxin, we can safely conclude that extraction of foxgloves (standard, super or slurry) will continue until such time as the medicinal and organic chemists provide better — cheaper, less toxic, more efficacious — alternatives. But that is likely to happen during the next decade.

The same general conclusion can be extended to other natural products, bleomycin or adriamycin, for example, for the skills and power of the medicinal chemists have now outstripped nature. Computer chemistry will speed this process.

Animal sources

Other natural medicines are extracted from animal tissues or animal cell cultures. Two kind of extracts are possible. The first is typified by insulin, where a useful chemical is produced from the glands of animals (cows or pigs) which are bred and killed for other purposes. Thus, insulin is a valuable by-product of a different industry. Fractions of different components are also made from human blood. A more macabre example is the extraction of human growth hormone from the glands of dead people. The second kind of extract is the basis of the vaccine industry, which has tradition-

ally relied on growing bacteria or viruses in enormous quantities, treating them in such a way that their pathological activity is lost but their capacity to immunize (immunogenicity) retained. When the resultant "soup" is injected into animal or man, the immune system reacts defensively to the antigenic content (usually a protein or carbohydrate) in such a way that the whole organism is prepared to defend itself against invasion by the living bacteria or virus. The soup sometimes contains live attenuated viruses which lead to actual viral replication in the host, thus eliciting a productive antibody response (as in mumps, measles, rubella, polio, yellow fever); such vaccines could not easily be supplanted by synthetic approaches, and they exhibit potent adjuvant activity and long-term protection.

Somewhere between the two examples are substances like interferon, interleukins, urokinase and tissue plasminogen activator with actual or potential therapeutic actions. These can be produced in quantity from human cells grown *in vitro*. For example, human α -interferon can be produced in commercial quantities by deep cell culture methods pioneered by The Wellcome Foundation.

Traditionally prepared vaccines would never meet the standards required of synthetic medicines, with the demand that the inactive ingredients should be characterized and that the active principle should be more than 90 per cent (and sometimes more than 99 per cent) pure. The antigen or active ingredient of a vaccine may represent less than 1 per cent of the material in a mixture of hundreds of unknown by-products of the bacterial, viral and animal material used to generate them.

It is in this overall field of protein chemistry that genetic engineering offers to provide directly applicable products of therapeutic value and commercial reward. Large protein molecules are, at present, too complex for the synthetic chemist to tackle, so that the biosynthetic route offered by gene-splicing is enormously promising for the large-scale production of these proteins. Furthermore, proteins of animal origin often differ slightly in chemical structure from those of human origin, so that when given to man for replacement or other therapeutic use, they may be recognized as "foreign" and thus lead to unwanted reactions. For this reason, an additional attraction of the

gene-splicing route is that it can provide human proteins. Many of these, such as insulin, interferons and growth hormone, have already been successfully prepared and are either on the market or in clinical trial. The major challenges lie within a limited number of potential candidates from which only a few may offer significant medical value and commercial potential.

Gene products

We are now seeing the commercialization of insulin and interferons derived by gene-splicing techniques. Human insulin is already on the market. However, it is still not clear whether the investment that has been made to fulfill this specific purpose will ever be justified by sales of human insulin alone. Immunogenic intolerance to bovine or porcine insulin (requiring treatment with human material) is extremely rare, the synthetic insulin appears to produce immunological problems of its own and, in any case, the present pancreas-extracted insulins are cheap. The extraction from the pancreas of animals is not only a side-product of the meat industry, but has also become a multi-faceted industry in its own right, yielding enzymes as well as insulin; until the enzymes as well as the insulin are more cheaply available from gene-splicing techniques, the very slight differences in side effects predicted for human insulin may not provide the necessary commercial edge. Moreover, some of the goals of genetically engineered "new" or different proteins can also be achieved by chemical modification (by traditional techniques) of available proteins (such as bovine insulin). Human insulin derived from gene splicing may turn out to be a "loss leader".

Biosynthetic interferons could well be different. There are aspirations that if a gene-spliced interferon can be made pure and commercially viable, it may be used in conditions such as the common cold² and for other viral diseases, including influenza, although present experience suggests that the side effects may not be acceptable for most of the self-limiting and mild viral diseases. Given that more refractory medical conditions (such as hepatitis and solid tumours) respond to interferons, they may be particularly attractive targets for, unlike insulin, (1) they are not available in any single tissue or organ for simple extraction (they are not stored in tissues); (2), they have proved extremely difficult to purify in significant quantities; (3) there are several chemically distinct classes (and probably subclasses) of the hormone and, (4), large amounts are needed for clinical experimentation simply to determine their specific utility.

Other human proteins also in the sights of the genetic engineers include growth hormone (but will it be commercially viable?), serum albumin, urokinase, plasminogen activator, factor VIII and lymphokines (the potential applications of

which are presently unknown). Most such potential medicines will need to be injected because of their chemical nature. Drugs available orally will always have more appeal than drugs given by other routes. Simpler but equally effective small molecules capable of synthesis by chemists will always be more attractive than complex proteins, no matter how synthesized. It is for these (and other related) reasons that the biosynthetic route, including gene-splicing, will always be threatened by advances in biology and biochemistry and by chemical techniques. In fact in most spheres of activity, the genetic engineering approach to the biosynthesis of specific molecules is a prelude to (and will encourage) development of superior and less expensive alternatives by organic chemists and biochemists.

The genetic engineering approach, coupled with Milstein's monoclonal antibodies³ and affinity methods for protein purification⁴, already offers an alternative method for the characterization of proteinaceous hormones, transmitters and neuropeptides of potential therapeutic importance. Many such hormones (enkephalins, endorphins, interleukins, morphine, lymphokines, transforming peptides, macrocortin) have been recognized in the past few years, and there are clearly many more to come. The tools of the genetic engineer will provide reagents that will contribute greatly to this field. Not only will the new techniques provide large quantities of pure peptides for study, but they will also assist in identifying and isolating the targets, or receptors, of their action. New and important therapeutic avenues must arise from this work. Recombinant genetic approaches will be invaluable for the study of cellular control mechanisms. Furthermore, recent advances in understanding the nature, chemical structure and function of numerous receptors provide essential information for the medicinal chemist. The ultimate therapeutic value of highly active molecules such as interferon, insulin, growth factors and lymphokines lies not so much in their end-use as therapeutic agents, but more in what they teach us about the disease process. It is in this context of new knowledge and understanding that truly selective and rationally derived new therapeutic agents will arise. Thus, understanding of the mode of action of peptide transforming growth factors, and the expression and target activity of oncogenes, may ultimately lead to the selective control of the malignant state.

Vaccines

Genetic engineering offers many opportunities for the development of new and improved vaccines. Some, like foot and mouth disease vaccine (FMDV) and hepatitis, are near at hand. Others, like a human malaria vaccine, are within sight⁵. The targets range from an attack on present markets such as FMDV through a range of

achievable new human and animal vaccines against bacterial, viral and protozoal infections, to still hypothetical vaccines that might make sheep the size of elephants, or prevent pregnancy. Those who side with prophylactic rather than therapeutic medicine can only be strengthened by the reality of gene-splicing. The progress that may be expected in the vaccine field is shown in Fig. 1. We are through phases A and B and into phase C. To understand the further progress which is possible needs only an elementary understanding of protein chemistry. The amino acids from which proteins are strung together are each about the same chemical size (molecular weight about 150) as one of Dale's transmitters, such as histamine or adrenaline. While the smallest of the proteins (or peptides) of 1 to 50 amino acids, such as angiotensin, may retain a linear structure, larger proteins (several hundred amino acids) wrap up into a three-dimensional complex, each with its own geographical characteristics determined by the type and number of amino acids.

Although it was originally thought that the ability of foreign proteins to stimulate the body to react defensively by producing antibodies (their immunogenicity) was linked primarily to the shape or tertiary structure of the whole molecule, studies with plant and bacterial viruses have shown that antiviral activity can be elicited by a small synthetic peptide corresponding to a region of the antigen involved in virus neutralization⁶. This work has been extended to animal viruses to induce an immune response to FMDV using a small linear part (14 amino acids) of the protein antigen⁷. This suggests that "a small peptide may be able to adopt a conformation approximating that which it assumes in the virus particle". Similarly, total synthesis of a diphtheria vaccine based on fewer than 20 amino acid residues has been achieved⁸.

Whatever may be the conformation needed to elicit antibody activity with such peptides, these results imply enormous consequences for the production of antigens by gene splicing techniques. For a peptide consisting of a score of amino acids is well within the scope of synthesis by medicinal chemists, and so is potentially a synthetic chemical rather than a bio-synthetic chemical or a "biological". Clearly, should this work be predictive for other single protein vaccines, the transition from phase C to phase D (Fig. 1) could well take place in the next few years.

Ten years ago, the idea of a peptide carrying activity into the body via the oral route was totally unrealistic, simply because the gut is designed to break down and provide a protective impermeable barrier for larger molecules (with, say, molecular weight exceeding 1,500). Now, orally active molecules based on a peptide structure are becoming common, through the skilful insertion of "unnatural" substituents. Two examples will illustrate

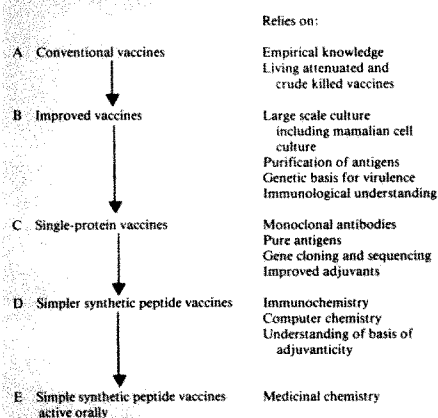


Fig. 1 The pattern of progress in vaccine production.

the point. The first is the development of the drug Captopril, an antihypertensive agent based upon inhibition of angiotensin converting enzyme. The first such inhibitors were peptides extracted from snake venom⁹. Knowledge of their structures led to the synthesis¹⁰ of a hybrid molecule which retained the required activity, but was orally active. The second example is in the field of enkephalins, where replacement of the naturally occurring L-amino acids (such as alanine) in the pentapeptide by the unnatural D-isomer¹¹ conferred a stability against enzyme degradation which allowed oral activity.

Thus, the transition from phase D to phase E (Fig. 1) is already theoretically possible. But a serious limitation to progress (even from B to C) is the need to strengthen the body's immunological recognition of and reaction to antigens by the use of adjuvants. This is easier to do in animals because adjuvants of a more toxic (or inflammatory) nature can be used. In man, anything worse than a sore arm for a few days would be unacceptable. For this reason, it is essential that better adjuvants be found. Perhaps even more important, we need to understand the mechanism of action of adjuvants in order to go to phase E. This has, surprisingly, been a generally refractory field of research.

Chemical synthesis

Virtually all new chemical entities of major therapeutic importance in the immediate past (such as propranolol, allopurinol, acyclovir, cimetidine) have arisen from chemical synthesis, and will do so in the future. There have recently been stupendous advances in biology and medicine, and numerous new molecular targets (enzymes, receptors, transmitters, mediators) are presently being identified as attractive and rational therapeutic points of leverage. At the same time, the ancillary tools are becoming ever more powerful. Furthermore, the automation of chemical reactions is allowing the synthesis of molecules of a type, and on a scale, unheard of even 20 years ago. This is especially true of the peptide field. On the one hand, the synthetic chemist is able to put together longer peptides, on the other,

the peptide needed to convey a specific antigenicity as a vaccine is getting smaller. It seems inevitable, therefore, that the medicinal chemist will eventually dominate prophylaxis as well as therapeutics.

Another enabling method that reinforces this conclusion is the computer chemistry or computer graphic technique¹². By visualizing the actual tertiary structure of receptors (also proteins), and the ways in which drugs interact with their active sites, the rate at which the chemist will be able deliberately to design heterocyclic molecules to fit into or modify such sites will be increased dramatically. These methods should yield standard principles applicable to the synthesis of unique custom-made molecules. For example, sufficient knowledge regarding the manner by which insulin (or interferon) interacts with its receptor at the molecular level should lead chemists to the design and synthesis of simpler, yet specific small molecules, not necessarily peptides, which will do the same (or a better) job as insulin (or interferon). The complex biological molecules will then no longer be needed as medicines.

Drug delivery

A new chemical entity, whether synthetic or biosynthetic, not only has to be active but also has to reach that part of the body where it is needed. Where an illness is associated with a particular organ (such as the liver or lungs), or with a discrete site (such as a solid tumour), it is advantageous to try to concentrate the drug in that tissue needing treatment, so that adverse effects on other tissues are minimized.

Simple ways of localizing or concentrating drugs have been known for many years. The inhalation of anti-asthmatic drugs theoretically takes substances directly to the organ concerned. A more exotic way of delivering drugs to a particular site or organ is to tie them to a messenger addressed to that organ. Antibodies directed at particular tissues have an obvious potential, and many groups are now searching for monoclonal antibodies that will attach specifically to cancer cells. A general antibody that reacts with cancer cells but not with normal cells will have not only diagnostic application but also the potential to carry a toxic message directed only at cancer cells. A further advance (if not a requirement, for selectivity) will be to direct the messengers to molecular entities on the cell surface which are normally destined to be internalized, or directly to the interior of selected cells, where they can do their damage.

This is an area of development in the biotechnology field of substantial importance. It is unlikely that, in the foreseeable future, medicinal chemists will find better messengers than can the immunologists. But the skills of the medicinal chemist will be needed to put the message into the hands of the specific chemical messengers and even to devise the

nature of the message, which may be radiochemical.

Future prospects

Over the next 20 years the various techniques associated with "genetic engineering" will infiltrate all fields of industry and science. Agriculture, energy supply and farming will all become more efficient. In science in general, and in those sciences applicable to the pharmaceutical industry in particular, there will be a substantial acceleration in the acquisition of knowledge of the working of different cells, and of the ways in which they communicate with each other. Monoclonal antibodies and gene cloning will also contribute significantly to the diagnosis and correction of genetic disorders. Importantly, the very knowledge which will be generated by the techniques will enable other disciplines, such as pharmacology, biochemistry and chemistry, to reap the fruits of progress.

At the same time, however, the synthetic chemical routes will eventually replace the biosynthetic routes offered by gene-splicing. For example, breakthroughs with synthetic chemical antiviral agents, such as Wellcome's new antiherpes drug, acyclovir¹³, will be followed by others that could make interferon obsolete. Similarly, insulin will be supplanted when there are discovered small molecules which mimic its action (as opioids, enkephalins and endorphins can resemble each other at receptor sites). Other simpler molecules will surely replace macrocortin, interleukins, endorphins, peptide hormones and so on.

From all this, it should be evident that the thrust of innovation depends on many disciplines working together. Creative biology and medicine must be the enabling force, by providing a body of knowledge which can be exploited by chemistry. In addition, the skills of the genetic engineer and the immunologist are essential not only to provide new (if temporary) prophylaxis and therapy but, much more importantly, to contribute to fundamental knowledge. For this reason, they should be integrated as closely as possible into the existing patterns of discovery rather than be isolated, as means to commercial ends, in their own compartments. □

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Who will clone a chromosome?

The cloning of factor VIII, on which most haemophiliacs depend, is a technical triumph without parallel, but has left important questions unanswered. They rest for others.

By sad coincidence, this week there may be special interest in the publication of four long articles (pp.326 *et seq.*) describing how two groups in the United States have been able to isolate the gene responsible for human factor VIII, one of the essential components of the blood-clotting system. The significance of this development is described on p.326 by Professor George Brownlee and Dr Charles Rizza.

They explain that Genentech and the Genetics Institute have been able to clone the gene for factor VIII, to work out its nucleotide sequence, to infer the way in which the corresponding very large protein molecule is put together from amino acids and even to devise a system by means of which factor VIII may eventually be manufactured in large quantities.

This is also the week in which people in Australia have been startled by the news of the death of at least three infants as a consequence of transfusion with blood now suspected of being contaminated with the virus (called HTLVIII) responsible for acquired immune deficiency syndrome (AIDS), and in which a British haemophilia patient died from what appear to have been similar causes, the contamination of supposedly pure factor VIII by the same virus.

Although people in the United States have been familiar with such tragic happenings for at least two years, the spread of this bad news is a chilling reminder both of the dangers to us all embodied by the virus responsible for AIDS and of the urgency of the need for a process for producing factor VIII that may be free from risks of that kind. It is especially cruel that people suffering from haemophilia, already a sufficient hazard to peace of mind, should now be exposed to a further risk over which they have literally no control. Whatever the commercial interests of those responsible for this week's reports, their fleetness of foot will earn full-throated congratulation. Such is the promise of what we call genetic engineering.

Unfortunately, this week's clutch of papers will also help to give that field a less enviable reputation. In the application of molecular biology to practical affairs, it is for the time being clear that bigger battalions move faster. So is there a danger, already explicit in high-energy physics, that the list of the names of the authors of a scientific article will be longer than a reasonably succinct listing of its

conclusions? Readers must judge for themselves, recognizing as they do so the extraordinary character of what has been accomplished.

Hitherto, even the natural site at which factor VIII is synthesized has not been known (but the liver was always a candidate), the protein molecule is the largest whose genetic origin has yet been characterized and the circumstance that the protein (but not, yet, the glycosylation parts that naturally go with it) can actually be manufactured on a laboratory scale would not have been thought possible even at the beginning of this year.

Two sceptics' questions nevertheless arise, the first of which is whether, in the single-minded pursuit of something of undoubted value, those responsible for this staggering exploit may have neglected tantalizing scientific issues that could in the end turn out to be more interesting. And, perhaps, even more important.

Here is an example. To many readers, the most arresting feature of the stack of papers now published will be the information, mentioned almost in passing by both groups, that part of the factor VIII molecule now synthesized resembles in its amino acid sequence part of the molecule of a quite unrelated protein, the plasma protein called ceruloplasmin, whose function is for the time being largely unknown. Ceruloplasmin has two unambiguous properties — it is blue in colour (which is what its name implies) and it binds copper atoms (between four and six per molecule). But properly supplied with copper, ceruloplasmin is an oxidizing enzyme. The common guess is that its function in the human plasma is to convert ferrous (Fe^{2+}) into ferric (Fe^{3+}) ions. But nobody seems to have conclusive proof.

So why, teleologically speaking, should factor VIII resemble such a molecule? There are obviously several candidate answers. Perhaps factor VIII, like ceruloplasmin, also binds copper atoms in ways that are important to its function. (Molecules of factor V, the analogous component of the blood-clotting system, have at least one copper-binding site.) As yet, it seems, nobody has checked.

It is also, however, possible that evolution, always ingenious, may have hit on a way of using a pre-existing molecular structure (ceruloplasmin) as a way of helping blood to clot rather than its normal function, for the sake of argument that defying the second law of thermo-

dynamics, by banishing ferrous ions when they would normally occur. (Ferrous ions are an obvious threat to living things; ferrous chloride is virtually insoluble, they form free radicals easily.) The trouble is that is nobody, even now (and after reading pp.326 *et seq.*) can know whether factor VIII is a copper binding protein. Factor V is, but that could be a different matter.

In normal times, if the field were stagnant, these speculations would have been suitable material for a successful grant application (or several). So too would have been some of the other questions not merely unanswered but unasked by pp.326 *et seq.* Why (or, rather, how) does it come about that such a basic system as that responsible for the clotting of blood should require not one enzyme but several? (Because evolution has found it to be worthwhile, at the margin as the economists would say, to win a little extra efficiency at the cost of a great deal of extra elaboration, perhaps because the molecules required for these marginal purposes were already in essence available.) Why are the components of the blood-clotting system distributed so widely on the human chromosomes? (The same answer applies; they are where they happened to be, so to speak.)

But how does factor VIII function? What is the mechanism by which, in human plasma (and in association with another shadowy plasma protein, von Willebrand's factor) it catalyses the transformation of factor Xa? Nobody has begun to guess what may be at the bottom of this process. Nobody can be blamed, for even a few months ago the question would have seemed insufferably impertinent. Yet it needs to be answered, and quickly.

That raises the second question about the balance of this triumphant effort in the cloning of factor VIII that may yet come round to haunt the cloners — is it feasible to think of manufacturing by the kilo a molecule as large as that of factor VIII without risking that it will emerge corrupted, or as bad, ineffective, from the production line? Obviously, the genetic engineers would prefer to be manufacturing a simpler molecule, one related to the molecule whose structure had now been described, as a few amino acids in an antigenic molecule may represent the biological activity of the entire structure. Will that be feasible? Time, at the worst, will tell. Otherwise, a little research may make the question answerable.

John Maddox

Biotechnology

Clotting factor VIII cloned

from George G. Brownlee and Charles Rizza

ONE of the most exciting and ambitious goals of the biotechnology industry has been achieved. In papers that begin on page 326 of this issue, both Genentech, assisted by Tuddenham and colleagues of the Royal Free Hospital, London, and Genetics Institute, assisted by Fass and Knutson of the Mayo Clinic, Rochester, report the cloning of DNA for human blood clotting factor VIII:C and the production of the factor from cloned DNA expressed in mammalian cell lines.

Clotting factor VIII:C is the name given to the protein that is absent or defective in patients with classical haemophilia (haemophilia A), a sex-linked bleeding disorder of about 20 per 100,000 males. In the blood stream, factor VIII:C is associated with another protein, known as von Willebrand protein or factor VIII-related antigen, but factor VIII:C is solely responsible for the clotting activity of the complex (which is loosely termed factor VIII). Injection of factor VIII obtained from donor blood allows haemophiliacs to be successfully managed. But, unfortunately, the factor VIII preparations in use at the moment carry a high risk of transmitting blood-borne viruses such as non-A, non-B hepatitis virus. The virus that is thought to cause AIDS may also contaminate preparations and, although relatively few haemophiliacs have been affected so far, the fear of the disease has caused great anxiety to patients, their families and their doctors. A genetically-engineered factor VIII:C derived from a source other than blood should be free of such viruses. It should also be cheaper.

Both groups have used similar methods for the DNA cloning and expression of factor VIII:C and report very similar results. Oligonucleotide probes were synthesized on the basis of small sections of the amino acid sequence of purified factor VIII:C (either pig or human in origin), and used to screen an appropriate library of genomic clones to identify part of the factor VIII:C gene. The gene itself turned out to be extraordinarily long (180 kilobases), to have 26 exons and to occupy an estimated 0.1 per cent of the X chromosome (see Gitschier *et al.* on page 326 for further details).

For expression of protein it was necessary to isolate cDNA clones. These were obtained either from a human T-cell hybridoma line (Genentech) or from human liver (Genetics Institute) and sequence analyses allowed the derivation of the entire 2,332 amino acid sequences of the mature protein. Both groups (but see Vohar *et al.* on page 337 for an extended discussion), note that the protein is highly glycosylated and has an obvious domain

structure with predictable homology to another clotting factor, factor V, but also an unpredicted homology to ceruloplasmin — a serum protein believed to be involved in copper ion transport in the blood. The protein sequence also allows the definition of the main sites that are cleaved by thrombin in the process of activation of factor VIII:C.

The expression of factor VIII:C in mammalian cells required the reconstruction of a full length cDNA clone and its attachment to a viral promoter sequence. When this construction was introduced into either a hamster kidney cell line (Genentech) or a monkey kidney cell line (Genetics Institute), a human factor VIII:C-like activity was secreted into the media in which the cells were grown. The concentrations of factor VIII:C-like activity in the media were about one per cent (Genetics Institute) or seven per cent (Genentech) of the normal plasma concentration as assayed by a sensitive biochemical test based on the activation of factor X and hydrolysis of a chromogenic substrate. Furthermore, both groups showed that the secreted activity (further purified by affinity chromatography at Genentech) was able to correct the clotting time of plasma from a haemophiliac. Evidence that these assays are indeed a measure of factor VIII:C and not of some other clotting factor or non-specific activity, was provided by two additional tests carried out by both groups. In the first, the clotting activity was shown to be increased by

thrombin, a normal physiological activator of factor VIII:C. In the second, it was shown to be inhibited by specific factor VIII:C antibodies. The Genentech group also showed that, like authentic factor VIII:C, their substance bound reversibly to the von Willebrand protein immobilized on an affinity column and that its activity was destroyed by protein C — a serine protease of plasma that can inactivate factor VIII:C.

What is the outlook for treatment of haemophiliacs? It is clear from previous experience with genetically-engineered products that we must anticipate some delay, perhaps 3–5 years, before a safe, effective and clinically-tested product is on the market. The expression of factor VIII:C reported by both groups is very low and it is probable that more effective vectors or host-cell systems will have to be devised. Effective large-scale tissue culture and purification methods will also have to be developed. These may differ from existing procedures for purification of factor VIII from blood. We emphasize the need for a high purity product to guard against the possibility of any pathogenic material from the culture being introduced into haemophiliacs. It would be unacceptable to eliminate the risk of AIDS but substitute a new hazard. There remains the possibility that the genetically-engineered factor VIII:C, although of proven activity in a test-tube assay, may not be as effective *in vivo*. This can only be adequately tested by clinical trials of the product, the outcome of which must be eagerly awaited. □

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Astrophysics

Evolution of star-planet systems

from Geoffrey Bath

A PROBLEM at the heart of stellar astrophysics is the role of angular momentum in the evolution of stars. This statement may seem controversial, given the profound success of stellar evolution theory in describing quantitatively the nucleosynthesis of the elements and the evolutionary transformation in the structure of stars as they perform their function as cosmic alchemists. The theory of stellar evolution has been largely developed without involving rotation in any major way. But a recent paper by M. Livio and N. Soker (*Mon. Not. R. astr. Soc.* **208**, 763; 1984) raises the dramatic possibility that the evolutionary fate of many single stars accompanied by planets could be both binary star systems and exploding variables.

Certainly, in the case of the Sun, present angular momentum, if rotation is uniform,

is quite unimportant in determining structure or immediate evolutionary future. The Sun cannot, however, be considered as an isolated unit; it is only one part — albeit by far the most massive part — of the Solar System. The angular momentum of the planets is sixty times greater than that of the Sun's own, assuming solid-body rotation in the inner core. Furthermore, the initial solar nebula, with a minimum mass of some 3 per cent of the Sun's mass, must have possessed an even greater fraction of angular momentum.

In the case of binary stars, angular momentum plays a very important part in determining their present binary state and subsequent evolution. In binaries, angular momentum decreases with decreasing separation. Even for W Ursae Majoris

stars, however, in which two relatively low-mass and approximately main-sequence stars are orbiting in physical contact, exchanging energy and surrounded by a common envelope, the angular momentum is hundreds of times greater than that of the Sun. Since more than one-half of all stars in the Galaxy are binaries, then if potential planetary systems around single stars are ignored, the significance of angular momentum is evident.

It was angular momentum considerations of these sorts that led Otto Struve in the 1940s to make the radical hypothesis of an evolutionary sequence that goes from giant binaries to dwarf binaries, and then to single stars with accompanying planets. He envisaged this being a result of the progressive dissipation of energy and loss of angular momentum associated with turbulence and differential rotation that von Weizacker was then advocating. As a general evolutionary theory Struve's notion has to be rejected, largely on the grounds that the dissipation time scales are too long in general to compete with nucleosynthesis as the main process of stellar ageing. There is incontrovertible evidence, however, that at least some stages of Struve's sequence have been significant and account for the formation of at least one important class of stars, the cataclysmic variables — though how is still a matter of debate.

Cataclysmic variables are binaries composed of a main-sequence star and white dwarf companion. The stars are so close that material from the surface layers of the distorted, pear-shaped, but otherwise apparently normal main-sequence star is being gravitationally captured by the white dwarf. Cataclysmic eruptions characterize these stars as novae and related exploding stars, generated both by unstable bursts in the mass transfer process and through runaway nuclear fusion reactions in the accreted hydrogen-rich material on the white dwarf surface.

One outstanding problem is the origin of these variables. Why is a white dwarf, which is formed in the deep interior of a red giant, now so close to a main-sequence star? In order to allow its precursor giant to form, the separation there must have been > 200 solar radii ($R_{\odot}$), whereas now it is $\approx 2 R_{\odot}$. Evidently either angular momentum has been lost, and lost relatively rapidly, or an extreme mass ratio originally allowed large separation with the present quota of angular momentum.

Livio and Soker have been exploring in some detail the second hypothesis, as originally advanced by P. Eggleton. They have examined the interaction of a planet immersed within a red giant's extended envelope. As it orbits within the envelope, tides are raised, viscous drag leads to dissipation and loss of orbital energy, and in certain conditions mass may be accreted efficiently. As a result, the planet acts as a catalyst, spiralling in through the envelope and accreting some fraction of the stellar

material as it does so. Livio and Soker find that the final mass of the planet is almost independent of the initial planet's 'seed' mass and is $\approx 0.14 M_{\odot}$, whereas the separation falls from $400 R_{\odot}$ to the range $8.6\text{--}0.5 R_{\odot}$. In this model for the creation of cataclysmic variables, loss of angular momentum is not invoked. Rather it is the change from the extreme mass ratio of a planet-giant system to the less extreme dwarf-white dwarf system that drives the two components together.

The present treatment is limited to somewhat *ad hoc* envelope conditions and assumptions of angular momentum conservation. Nonetheless, the principles by which a planet or intermediate dwarf may be converted into a low-mass main-sequence star, via the stripping of a red giant envelope to expose the degenerate core, are clearly revealed. More recent work by the same group shows that the

same features are present when the giant envelope reacts self-consistently to the spiralling process. If this is proved by future observational tests to be the origin of cataclysmic variables, Struve's ideas will have found some support, but with the important difference that the evolution from a wide planetary system to close interacting binary is in the opposite sense to Struve's early thesis. But then stellar evolution of single stars was long thought, erroneously, to proceed from red giant to main-sequence dwarf. With the idea that the fate of our own Solar System might conceivably lie as a cataclysmic nova, a new possible future for the Sun has to be confronted. □

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Molecular biology

Multiple levels of gene control in eukaryotic cells

from Geoffrey North

How can a gene be regulated by steroid hormones in some cells of a chicken and not in others, in which it is either expressed constitutively or not at all, even though both categories of cells may contain perfectly functional steroid hormone receptors? And how is the level of expression of immunoglobulin heavy-chain genes increased approximately 30-fold when B lymphocytes differentiate into antibody-secreting plasma cells? The possible answers to these puzzles, which emerged at a recent meeting*, illustrate how multiple levels of control combine to ensure that the right genes of eukaryotes are expressed at the right times, in the right amounts and in the appropriate cells.

The pattern of immunoglobulin heavy-chain gene expression in B cells has been presumed to be at least partially accounted for by the 'enhancers' that were discovered last year between the joining (J) and first constant (C_{μ}) exons of both human and mouse genes¹⁻³. These were the first gene-regulatory sequences of eukaryotes to be discovered that manifest cell-type specificity. Typically for enhancers, they can stimulate transcription of a gene whichever orientation they are in and at variable distances upstream or downstream from the gene. However, as W. Schaffner (Institute für Molekularbiologie, Zürich) explained in Heidelberg, this clearly cannot be the whole story.

The original evidence that these enhancers are cell-type specific came from experiments in which they were linked in

various ways to a test gene, such as that encoding β -globin, which was subsequently shown to be efficiently expressed in transfected B cells but not in other cell-types, such as fibroblasts or epithelial cells. However, Schaffner reported that a test gene (the T-antigen gene) linked to a mouse immunoglobulin heavy chain gene enhancer is expressed as efficiently in pre-B and B cells as in plasma cells, even though the level of expression of the endogenous immunoglobulin heavy-chain gene increases dramatically as B cells are stimulated to differentiate into plasma cells.

This observation has led Schaffner and his colleagues to investigate just how the rates of transcription of the immunoglobulin heavy-chain gene compare in pre-B, B and plasma cells. Surprisingly, the rates turn out to be identical at each stage of B-cell differentiation, so the higher level of immunoglobulin heavy chain mRNA in plasma cells than in pre-B and B cells must be the result of some post-transcriptional control mechanism, such as a change in RNA stability.

Further support for this inference comes from the observation reported by M. Neuberger (MRC Laboratory of Molecular Biology, Cambridge) that when the immunoglobulin heavy-chain gene itself is reintroduced into pre-B, B or plasma cells under the control of its own enhancer, its level of expression reflects that of the endogenous gene. Neuberger also reported evidence that the enhancer is not the only component of the immunoglobulin heavy-chain gene to manifest cell-type specificity: the coding region of a β -globin gene linked to the promoter of the immunoglobulin

*'The Control of Transcription in Eukaryotes', 10th annual EMBO symposium, 24-27 September 1984, European Molecular Biology Laboratory, Heidelberg, FRG.

heavy-chain gene (which flanks the 5' side of the variable, *V*, region) is properly transcribed only in B cells, even when linked to the SV40 enhancer, which is known to work in many different cell-types. Neuberger suggested that this specificity may be conferred by the conserved sequence that has been found upstream of all immunoglobulin gene *V* regions sequenced to date⁶.

The precise role of enhancer sequences in immunoglobulin gene expression has also been brought into question by two observations. First, the λ light-chain gene does not seem to have one, and second the translocations that frequently have juxtaposed immunoglobulin genes with the *c-myc* gene in B-cell malignancies more often than not have removed the enhancer to the reciprocal translocated chromosome (see ref. 7 and references therein). The implication of the latter observation is that the immunoglobulin gene enhancer cannot be directly responsible for the deregulation of the *c-myc* gene that is thought to have played a key role in these types of cellular transformation⁷.

A similar apparent lack of a requirement for an enhancer is shown by the continued heavy-chain gene expression after spontaneous deletion of regions of the gene that include the enhancer^{8,9}. For one of these cases, that of a δ heavy-chain-producing mouse hybridoma cell line⁹, Schaffner's group has preliminary evidence that the cloned enhancerless gene must be linked to a new enhancer in order to be expressed when it is reintroduced into B cells. On that basis, Schaffner suggests that enhancers are perhaps required for the establishment of transcription, but are not essential for its maintenance. As Schaffner pointed out, this could account for the apparent lack of requirement for an enhancer for the deregulation of translocated *myc* genes.

If this is so, then why are genes that are turned on by steroid hormones turned off again when the hormone is withdrawn, given that the sequences upstream of genes that confer steroid-responsiveness now seem to be just like the inducible enhancers^{10,11}? A possible clue may be in some results reported in Heidelberg by P. Chambon (Institut de Chimie Biologique, Strasbourg).

The principal aim of the experiments reported by Chambon, like those reported by G. Schutz (Institute of Cell and Tumor Biology, Heidelberg) was, however, to answer the puzzle posed in the first sentence of this article — how can one account for the different patterns of expression of steroid-inducible genes in different cell types, all of which contain the appropriate steroid hormone receptors.

For example, the chicken ovalbumin gene is induced by oestrogen in oviduct cells, in which the vitellogenin gene is permanently inactive, whereas in liver cells the ovalbumin gene is permanently inactive and the vitellogenin gene is oestrogen-inducible. Perhaps even more puzzling is

the chicken lysozyme gene, which can be induced by oestrogen, progesterone, testosterone or glucocorticoids in oviduct cells, but is permanently inactive in fibroblasts (which contain glucocorticoid receptors) and is expressed constitutively, albeit at a low level, in macrophages.

From an analysis of the expression in cultured cells of microinjected hybrid genes comprising the SV40 T-antigen coding region linked to flanking sequences of the chicken ovalbumin genes (Chambon) or lysozyme genes (Schutz), it seems that, in addition to hormone-responsive sequences, the genes are controlled by other cell-type specific sequences that further restrict their expression. However, in view of the observation of Chambon's group that the ovalbumin flanking sequences allow constitutive gene expression in injected liver cells, the inactivity of the endogenous gene in liver cells remains a puzzle. Chambon suggests the answer may lie in the establishment of domains of permanently inactive and potentially active chromatin during development.

Chambon also reported that hybrid genes containing up to 295 base pairs of the upstream flanking sequences of the ovalbumin gene are constitutively expressed in oviduct and liver cells, but that if 420 base pairs of flanking sequence are used, expression is blocked in oviduct, but not in liver cells. This block can be overcome by steroid hormones or by including an SV40 enhancer element in the microinjected construct. On the basis of these data, Chambon postulates that oviduct cells contain a repressor that recognizes the upstream 'blocking sequence' and keeps the level of expression of the ovalbumin gene minimally low in the absence of hormone. Could it be that such a repressor also has the role of re-establishing a requirement for enhancer-induction following hormone withdrawal?

The notion that enhancers may be required only for the establishment, and not for the maintenance, of a state of transcription is, however, somewhat difficult to reconcile with a model put forward by K. Yamamoto (University of California, San Francisco) to account for some recent results obtained by his group and which may also provide an explanation of some of the remaining puzzles of the regulation of steroid-inducible genes. In essence, Yamamoto reported that pairs of different enhancer sequences in combination can have synergistic effects on the genes to which they are linked, and that these effects depend on the precise nature of the promoter from which transcription of the gene is initiated.

Yamamoto suggests that these observations can be explained if different enhancers stimulate the supply to the promoter of different specific transcription factors, the synergism resulting from a change in the factor that is rate-limiting for transcription in the presence of one or

other, or both, the different types of enhancer. Following this line of argument, then, the failure of steroid hormones to increase the low constitutive level of lysozyme gene expression in macrophages could be due to the absence of some other factor which is necessary, though not sufficient, to enhance transcription.

This is all speculation, however, and in truth we still have little idea how even steroid hormone-induced enhancers activate transcription. Even the role of the steroid hormone is still obscure for, as M. Beato (Institut für Physiologische Chemie, Marburg) and W. Schrader (Texas Medical Center, Texas) explained, although the presence of hormone is required for the stimulation of transcription, the hormone-receptors bind quite happily to their specific sequences in the absence of hormone. Hopes that some of this ignorance might soon be dispelled were raised in Heidelberg by Yamamoto's news that his group has cloned cDNAs encoding the rat glucocorticoid receptor. □

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Geoffrey North is an assistant editor of *Nature*.



100 years ago BACTERIOLOGY

AMONG the most striking of the recent rapid advances of science is the development of what we may term bacteriology. While some of this work has been done in this country, by far the greater part has been done abroad, more especially in Germany and France, where its importance is recognised, and where special facilities are afforded by the Governments and various public bodies. In Germany especially, besides the laboratory, supported by the Government, in which Dr Koch works, a number of similar institutions are being established throughout the country; and in France the laboratories of Pasteur and others are established and supported by the Government and by various municipal authorities, every facility for carrying on these researches, and the necessary funds, being provided. In this country, on the other hand, there is no laboratory of the kind, and what work has been done has been by individual investigators working at their own expense and often without suitable accommodation.

From *Nature* **31**, 49, 20 November 1884.

Carbon cycle

Surprise from the shallows

from M. Whitfield

A RARE new insight into the workings of the carbon dioxide cycle is offered by the paper of Byrne *et al.* on page 321 of this issue and its companion, from the same group, which is published in this week's *Science* (226, 1074; 1984). In suggesting, from observations in the North Pacific, that the flux of calcium carbonate shells from the surface layers into the deep oceans has been grossly underestimated, these papers imply a significant shift in our understanding of the oceanic carbon dioxide system.

From an analysis of the particles that settled in large conical traps, deployed at various depths for only a day or so, Byrne and his colleagues were able to show for the first time that pteropod shells, which are made of aragonite — the most soluble form of oceanic calcium carbonate — contribute a flux of calcium carbonate to the deep ocean which is at least as great as that provided by the calcite shells of the foraminifera and coccolithophorids. Furthermore they show that, unlike calcite shells which fall into the sediment and then redissolve only slowly, most of the pteropod shells dissolve in the water column. Earlier measurements had been inconclusive; tow-net sampling had allowed no estimate to be made of the settling rate of pteropods, and long-term sediment traps had inadequately registered the presence of pteropods because their shells had dissolved in the weeks or months before the traps were recovered.

As the shells settle out of the surface layers into deeper water, they resist progressively more corrosive conditions until reaching their lysocline, where they begin to dissolve. Typical lysocline depths for the North Atlantic are 3.2 km for aragonite and 4.6 km for calcite. For the older and more acidic deep waters of the North Pacific, the lysocline depths are typically 0.6 km for aragonite and 0.8 km for calcite. Although calcium carbonate dissolves at and below the lysocline it can still be found in sediments down to the compensation depth, where rates of settling and dissolution just balance.

For each mole of calcium carbonate dissolved, one mole of carbon dioxide is converted into bicarbonate (resulting in an increase in oceanic alkalinity). The new findings make it possible to establish a realistic alkalinity balance for the oceans. Moreover they suggest that the oceans might act as a more rapid and effective sink for fossil-fuel carbon dioxide than had been thought possible when attention was focussed on the dissolution of calcite from the ocean sediments. In that case the response time is measured in centuries or millennia because of the slow circulation of

the carbon dioxide-rich waters down to the compensation depth. To dissolve a significant amount of calcium carbonate, these acidic waters must penetrate the sediments, still further slowing down the process.

The new observations indicate that 5-20 per cent of the smaller pteropod shells will have dissolved by the time they have reached a depth of 1,000 m. This dissolution provides a ready explanation for the mid-water alkalinity maximum that is observed in North Pacific waters substantially supersaturated with respect to calcite, which has previously been ascribed to the possible, but rather unlikely, long-distance transport of high alkalinity water from the edges of the ocean basin.

To close the circle, it is also necessary to consider the enhancement of shell dissolution by the increasing oceanic concentration of carbon dioxide that is resulting from the burning of fossil fuel. The new observations make it clear that this carbon dioxide has only to penetrate to the aragonite lysocline for it to have an effect. Model calculations suggest that it has

already reached a depth of 300 m — close to the aragonite lysocline in the North Pacific — as the result of the slow downward mixing of surface waters. If the dissolution of pteropod shells in relatively shallow waters is enhanced in this way, there will be a corresponding reduction in the alkalinity of the deeper water. In turn, this will accelerate the dissolution of the calcite shells and the larger aragonite shells in the sediment. Furthermore, as the falling shells begin to dissolve, their rate of fall will be reduced and hence their dissolution will become progressively more complete.

On a global basis, this process could accommodate up to one fifth of the current flux of fossil-fuel carbon dioxide and would therefore be much more important than the dissolution of shallow-water, magnesium-rich calcites that has previously been considered the best candidate to account for any rapid response. As oceanic carbon-cycle models become sufficiently sophisticated to incorporate biological processes in a realistic fashion, it will be instructive to see to what extent the oceanic uptake of fossil-fuel carbon dioxide will be influenced by the shift in the alkalinity balance implied by the flux of pteropod shells to the deep ocean and by their tendency to dissolve in transit. □

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Cell biology

A spectrum of spectrins

from A.J. Baines

SOME two years ago I alluded in these columns to the discovery of analogues of the red cell membrane skeleton protein, spectrin, in unexpected places¹. Since then they have been looked for and found — at least by the somewhat rudimentary technique of immunological cross-reaction — in almost every kind of cell that the imagination of cell biologists can run to.

The outsider, trying to follow the trail of discovery, is apt to be put off the scent by a multiplicity of terminologies. Not only are several names (tissue spectrin, caldesmon, fodrin) still in use for the spectrin-like proteins of non-erythroid cells, but we now have multiple names for their subunits too. For instance, the smaller subunit of brain spectrin, because it binds to the membrane-associated retaining protein, ankyrin, and hybridizes with the larger, alpha subunit of either brain or red cell spectrin, is sometimes termed beta by analogy with the erythrocyte beta chain (for example ref. 2). On the other hand, its electrophoretic mobility in SDS differs from that of erythrocyte beta chain and peptide maps of the two are quite distinct, so what seems to be the same peptide turns up elsewhere under the designation gamma (for example, ref. 3). Ultimately, the relationship between these polypeptides will need to be resolved by

direct comparison of sequences, using that of the greater part of the red cell spectrin molecule⁴ as the standard.

So what does spectrin do in different cells? In parallel with its tissue distribution, some indications of the cellular functions in which it may be involved have accrued. Originally it was supposed that spectrin and its associated proteins, which together make up the red cell membrane skeleton, would perform much the same function in, say, a brain cell as they would in a red cell. Thus it was conceived that a plasma membrane needed a supporting cytoskeletal apparatus to prevent its disintegration, although, to be sure, no brain cell has to withstand the rigours endured by the red cell in circulation.

Complicating this attractively simple model has been the discovery that tissue spectrins are able to enter into associations with proteins not present in the mature red cell, as well as being able to bind protein 4.1, actin, ankyrin and calmodulin. They can bundle microtubules⁵ and bind to at least one of the microtubule-associated proteins (tau)⁶, activities which may be reflected in the antigenic cross-reactivity of spectrin with MAP II, another microtubule-associated protein⁷. Brain ankyrin also binds to microtubules⁸, although in

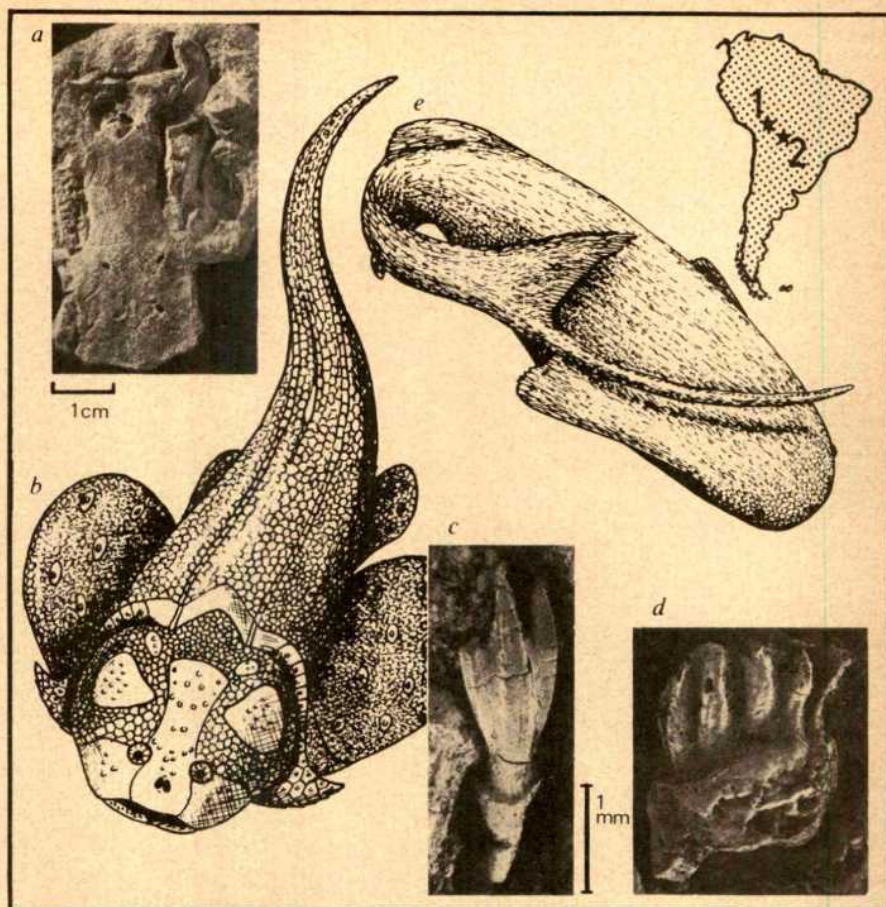
this case an interaction with tubulin dimer is suspected as well: here again there is cross-reaction with another microtubule-associated protein, MAP I⁹. Brain spectrin and ankyrin may indeed be the first examples of membrane-associated microtubule-binding proteins. An indication of an interaction between spectrin and intermediate filaments has come from evidence that when anti-spectrin antibodies are microinjected into cultured cells, not only is spectrin precipitated, but the organization of the intermediate filaments is disrupted¹⁰.

Support for the idea that spectrin is somehow involved in maintaining the physical state of membranes comes from the elegant immunofluorescence studies of Koenig and Repasky¹¹, who show that isolated axons are enriched in spectrin where they are not enclosed by a myelin sheath, that is at the nodes of Ranvier and the synapses. It therefore seems that spectrin is involved in the generation and maintenance of plasma membrane domains. However, this cannot be the whole story since the same group has discovered an association between spectrin, as well as calmodulin and F actin, and the rapidly moving axonal particles (motile varicosities), which are seen in axons regenerating *in vitro*¹². This suggests that the role of spectrin also encompasses the linkage of organelle membranes with cytoskeletal elements. Aunis and Perin¹³ have added a further twist by suggesting the implication of spectrin in chromaffin granule exocytosis. If spectrin is indeed involved in exocytosis then its concentration at the synapse could be taken to imply a role in neurotransmission. A further recent conjecture is that the cleavage of spectrin on post-synaptic membranes, triggered by depolarization-induced events, may be involved in memory storage¹⁴.

So in answer to the question of the cellular functions of spectrin, a general role is now discernible. Spectrin seems frequently to link cytoskeletal elements to plasma or other (for example exocytic) membranes, or to regulate that linkage. Cells may use the linkage in a variety of ways, including the generation and stabilization of plasma membrane domains, the facilitation of intracellular transport (leading in certain cases to exocytosis) and the regulation of the activities of transmembrane proteins. Watch this space. □

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Devonian vertebrates from South America

DEVONIAN vertebrates have been recorded from every continent except South America, where only a few fossils have been tentatively referred to this group (Janvier, P. *Program. intern. Correlat. geol.*, La Paz 44 1; 1978). The most convincing of these is a footprint of an amphibian, from the Devonian of Brazil. Lower and Middle Devonian sites of western and central Bolivia have now yielded scales of agnathans, spines and scales of acanthodians and sharks, and a skull and plates of a placoderm.

The figure illustrates two of the Devonian fishes we have excavated. The Middle Devonian Sicasica Formation, which extends south of La Paz on the Altiplano (site 1 on the map) yielded a well preserved braincase and the jaws of a rhenanid (a), a rare group of placoderms (armoured fishes) known from the Devonian of Europe and North America. There are close similarities between these fossils and a complete rhenanid, *Gemuendina stuerzii* (b) excavated from the Lower Devonian of Hunsrück, FRG. South-east of the Altiplano in the Chuquisaca district (site 2 on the map), the Lower Devonian Catavi Formation crops out along the Rio Grande at Seripona. There, a rich bone-bed has yielded remains of acanthodians, sharks and thelodonts, a group of jawless vertebrates. Two types of thelodont scales (c,d) from this locality may have belonged to a form resembling *Turinia* (e), known from the Lower Devonian of Scotland. The

scales also resemble those of a thelodont recently described from the Devonian of Australia, *Australolepis seddoni*. Similarly, peculiar shark spines from the locality recall those of *Antarctilamna*, from the Middle Devonian of Antarctica and Australia.

Moreover, large acanthodian spines from Seripona, as well as from several other Lower Devonian localities in Bolivia, display the same superficial ornamentation, with alternating broad and narrow ridges without nodes, as the spines of *Sinacanthus*, a large climatid acanthodian from the Middle Devonian of south China.

Any large-scale biogeographic conclusions would be premature. But at a time when the early history of the Pacific is being vigorously discussed by geophysicists and palaeobiogeographers (Nur, A. & Ben-Avraham, Z. in *Vicariance Biogeography, a Critique*, 341 (Columbia University Press, 1983), the Devonian fishes of South America should provide a valuable tool for estimating Palaeozoic trans-Pacific relationships, particularly since most of them lived very near to the continental shore.

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Cell activation

The 'basic' connection

from Walter F. Boron

CHARACTERIZATION of a new biological process almost always includes a description of how it is influenced by changes in pH. In the case of the processes controlled by the enzyme protein kinase C, the tables have been turned; current emphasis is placed on how activation of this kinase affects the pH within the cell (pH_i). Any process that alters pH_i is of potential interest inasmuch as a shift in pH_i could act as a signal to modulate a host of pH-sensitive processes. Activation of protein kinase C is of special interest, because it is believed to mediate the intracellular effects of a wide variety of extracellular signals. On page 371 of this issue Moolenaar *et al.* present evidence that activation of protein kinase C stimulates the plasma membrane Na-H exchanger — which is responsible for the exchange of sodium ions for hydrogen ions across the outer membrane of cells — and thereby leads to an elevation of pH_i in human fibroblasts, HeLa cells and mouse neuroblastoma cells¹. Grinstein *et al.* have reached similar conclusions in experiments on rat thymic lymphocytes². The implication of these data is that the intracellular alkalization induced by the activation of protein kinase C is an important step in the process by which certain extracellular signals elicit their physiological effects.

The Na-H exchanger ultimately affected by protein kinase C is normally involved in the active regulation of pH_i , a house-keeping function performed by all studied animal cells except the erythrocyte. The fundamental acid-base problem faced by these cells is their chronic tendency towards intracellular acidosis, usually caused by the passive influx of acids, such as H^+ , or efflux of bases, such as HCO_3^- , but sometimes caused by the metabolic generation of acid. Uncontrolled falls of pH_i are prevented by ion-transport systems that have the effect of extruding acid from the cell. The invertebrate system appears to exchange external Na^+ and HCO_3^- for internal Cl^- and H^+ , whereas most vertebrate cells use a transporter that exchanges external Na^+ for internal H^+ . Both acid-extrusion mechanisms are ideally suited to their role by being virtually inactive at high pH_i values, and progressively stimulated as pH_i falls below a certain 'threshold'³. This is at least in part due to an intracellular H^+ -binding modifier site, distinct from the site that binds H^+ for transport^{4,5}. The first evidence for modulation of a pH_i regulator was that cyclic AMP stimulates the $Na^+/HCO_3^- - Cl^-/H^+$ exchanger of barnacle muscle⁴. Part of the action of parathyroid hormone on the renal proximal tubule may be due to the inhibition of Na-H exchange by cyclic AMP⁵.

A possible role for Na-H exchange in the

regulation of cell growth by extracellular signals was suggested by experiments in which exposure of quiescent Swiss 3T3 cells to a combination of platelet-derived growth factor (PDGF), vasopressin and insulin, caused a Na^+ -dependent rise in pH_i of about 0.15, as well as a marked stimulation of Na^+ uptake⁷. Subsequently, epidermal growth factor (EGF) was shown to increase pH_i by 0.1–0.2 within ten minutes, but not in the presence of amiloride, an inhibitor of Na-H exchange^{8,9}. Activation of the quiescent cells culminates in cell division several hours later. The notion that an early rise of pH_i plays a role in triggering the cell into division is supported by the observation that the degree of intracellular alkalization induced by growth factors closely parallels the subsequent stimulation of DNA synthesis¹⁰. Thus, the Na-H exchanger, via its effect on pH_i , may be a critical regulator of cell growth.

The cascade of events by which growth factors stimulate Na-H exchange may be as follows: a growth factor, such as EGF¹¹ or PDGF¹², binds at the plasma membrane to a specific receptor (that also has the activity of phosphorylating proteins at tyrosyl residues). A consequence of the binding is the release of Ca^{2+} and increased breakdown of inositol phospholipids. The latter results in the production of diacylglycerol, a potent activator of protein kinase C¹³, which phosphorylates proteins, including the receptor for EGF (see ref. 14), at seryl and threonyl residues. Finally, as indicated by the new data of Moolenaar *et al.*¹, protein kinase C somehow stimulates the Na-H exchanger.

Certain phorbol ester tumour promoters that bear a structural similarity to diacylglycerol are commonly used to stimulate protein kinase C in laboratory investigations. Moolenaar *et al.* show that one such phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), but not a biologically inactive analogue, causes pH_i to increase by about 0.15 within ten minutes, much as it changes after direct stimulation of fibroblasts by polypeptide growth factors. The TPA-induced increase of pH_i is Na^+ dependent and is blocked by a potent amiloride analogue, indicating that it is caused by stimulation of a Na-H exchanger. Furthermore, TPA does not induce a pH_i increase in fibroblasts that have already been stimulated by PDGF. Inasmuch as TPA bypasses the growth-factor receptor/kinase step of the proposed cascade, a simplistic view would be that stimulation of the Na-H exchanger does not require the release of either Ca^{2+} or diacylglycerol *per se*, but only the activation of protein kinase C. Indeed, the

observations of Moolenaar *et al.* on Na-H exchange, as well as those of others on secretory processes (see ref. 15), indicate that TPA can produce its pharmacological effect without a measurable rise of intracellular Ca^{2+} .

The simplest explanation for the effects of TPA on pH_i , and the one proposed by Moolenaar *et al.*, is that protein kinase C directly phosphorylates the Na-H exchanger. This remains to be established. The kinetic effect of TPA on the Na-H exchanger could be to increase its pH_i threshold — that is to increase the value to which pH_i must rise before the Na-H exchanger shuts off — and/or to increase its sensitivity to acidosis once pH_i falls below the threshold. Moolenaar *et al.* favour the former. This is an attractive possibility, although it is easy to imagine how phosphorylation near the intracellular modifier site could alter either the pH_i threshold or the sensitivity of the Na-H exchanger.

Finally, it is intriguing to speculate on the possible physiological role of the stimulated Na-H exchanger. Most of the experiments on the effects of growth factors on pH_i have been conducted in HCO_3^- -free media. Elsewhere, Moolenaar *et al.* have reported that unstimulated human fibroblasts do not have a detectable HCO_3^- transport system¹⁶. However, L'Allemain *et al.*¹⁰ report that, whereas a functional Na-H exchanger is necessary for stimulating the mitogenesis of Chinese hamster lung fibroblasts that are incubated in HCO_3^- -free media, it is not necessary when the cells are in HCO_3^- -containing media. Is the Na-H exchanger in these hamster cells functionally replaced by a HCO_3^- transport system? Other, more general questions arise. Is a sudden increase of pH_i really necessary to trigger cell growth? Or do growth factors stimulate Na-H exchange for other reasons, such as to enhance the housekeeping ability of the cell to regulate pH_i in anticipation of the increased requirements of the cell on metabolic activation? □

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Territory, size and weight gain

SIR — A recent paper by Carpenter *et al.*¹, on territory size and weight gain, has been received with enthusiasm by Diamond². One must share his attitude towards their elegant experimental technique for weighing birds undisturbed in the wild. However, I believe more caution should be exercised with regard to their results and interpretation. Diamond quotes the example of bird WP whose rate of weight gain was measured at three territory sizes and found to be greatest at the intermediate and final size. "The results with WP and other rufous hummingbirds", Diamond says, "reveal that the birds adjust their territory size until they have maximized the rate of weight gain". But WP was in fact the only individual bird observed to have a higher rate of weight gain for an intermediate territory size and even in this case there is no direct evidence that the rate of weight gain achieved was a maximum. The justification for concluding that the birds did maximize their energy input was that in four out of five cases the highest weight gains achieved were on the last day or days of the birds' stay on the study site. Clearly this only demonstrates a level at which birds no longer attempt to increase their net energy input, not that that level is a maximum. The argument for a general "humped" relationship between territory size and weight gain is that some birds showed increases in weight gain with increasing territory size and some with decreasing territory size — a much weaker case than that implied by using the example of WP alone.

Alternative explanations for the observations are possible. For example, suppose that the rate of weight gain is related to the bird's body weight. The observed increase in rate of gain towards the end of each bird's stay is predicted, but is independent of territory size. This would also explain the unusually high rate of weight gain of bird BGR — the bird with the highest initial body weight.

A further problem arises with the proposed adjustment of territory size by "trial and error". Suppose a bird has a sub-optimal energy gain from its initial territory size. Given a bell-shaped relationship between territory size and weight gain, how does the bird decide in which direction to alter its territory size? Even after one alteration made in a random direction the bird still has no good grounds on which to make its next decision.

Apart from the valuable technical innovation, the most important point which comes out of this work is, surely, that the birds do not behave optimally. On arrival at the study site, they do not know and cannot know what the optimum territory is. It depends on many unknown factors, such as nectar production by the flowers and the number and quality of the birds'

neighbours. They spend at least the first few days, if not all of their stay, in territories that are either too large or too small and the best they can do is to try to increase their efficiency as time goes on.

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Pigeons, canaries and problem-solving

SIR — I regret that I must question the accuracy of Pastore's¹ recent letter concerning my report of problem-solving behaviour in pigeons². He suggests that I was remiss in not citing his early studies in which a canary was confronted with several Köhler-type problems. He implies that the canary solved both Köhler's one-box problem and Köhler's stacking problems in an "insightful" fashion, but the original reports of this work^{3,4} do not confirm that.

The single canary that Pastore confronted with a variant of the one-box problem required 25 reinforced trials before it could move its box (a cardboard "prism") smoothly to the correct position on the floor of the chamber. So the behaviour that superficially resembled that of Köhler's chimps was, as Pastore originally reported, learned in virtually the same haphazard fashion as the escapes of Thorndike's⁵ cats. Ironically, the performance of the canary was exactly that which Köhler dismissed as mere "trial and error". By no reasonable criteria could the canary's performance be considered "insightful".

On the stacking problem, in which stacking behaviour is also established haphazardly after many reinforced trials, Pastore³ originally reported that "in the crucial trial, when both prism and box were out of position, the canary seemed to be unable to stack them in a meaningful way. Actually, the canary did stack prism and box appropriately in only 10 of 100 trials" (p. 289). No mention is made of success in stacking a larger number of boxes, contrary to Pastore's¹ recent statement. A second canary could not complete even the preliminary stages of the experiment. Praxists and psychologists long ago passed judgment on these modest demonstrations: they are almost universally uncited in the literature relevant to my research.

In contrast, my colleagues and I reported a systematic study with 11 pigeons in which all 3 with relevant training histories solved the box-and-banana problem in a dramatic human-like fashion the first time they were confronted with it. The performances satisfied all of the traditional criteria of "insight": periods of apparent confusion were followed by sudden, rapid, and entirely appropriate performances. By

systematically varying the training histories of other birds, we also determined the possible contributions that a variety of different experiences had made to success in the problem. Finally, we offered a running account of the novel performances in terms of empirically validated principles.

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DNA sequence selection by eye

SIR — The recent correspondence concerning various ways of representing DNA sequences^{1,2} prompts me to describe a simple method for finding sequence overlaps by eye. Using the 4,361-nucleotide sequence of the plasmid pBR322 as a test system, it is possible with a little practice to locate a randomly selected sequence of about 100 nucleotides in less than 2 minutes.

The method requires four ballpoint pens of different colours and the sequence (or collection of sequences) with which the test sequence is to be compared is coded as follows. Every time the sequence AG (an arbitrary choice) occurs, the next base is coloured, the colour used depending on the next base again (G green, A blue, C black, and T red). Thus AGCT appears as a red C, AGGG appears as a green G and so forth. This process takes about 10 minutes per kilobase, but it only has to be done once for each new sequence determined.

To locate the test sequence, an AG is selected at random and about ten adjacent downstream bases are written with a sharp pencil on the edge of a piece of paper. The appropriate base adjacent to the G is coloured and the sequence is checked against each occurrence of that coloured letter, on average four times per kilobase.

If two AG sequences are close together on the test sequence, the pattern of two coloured letters separated by a certain number of bases makes searching faster and also highly specific. Such a pattern would only occur at random three times in the entire Epstein-Barr virus sequence.

If the sequence is not found, the complementary strand is searched by locating one or more CT sequences, writing down the ten or so downstream bases on the complementary strand and proceeding as before.

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Transformation by the p21^{ras} protein

SIR — Ralph¹ has questioned the validity of certain speculative comments of mine concerning the mechanisms by which p21^{ras} proteins transform mammalian cells. He has obviously misunderstood the point of my remarks.

In my article, I drew an analogy between new findings showing loss of GTPase activity in the transforming protein and the fixation of adenylate cyclase Gs protein in the GTP state after cholera toxin binding. In case it was not made clear in the article, perhaps I should state that there is no evidence of a functional link between the adenylate cyclase system and p21^{ras}, and therefore for a direct role of cyclic AMP regulation in *ras*-mediated transformation. Examples of some of the known effects of cholera toxin on cell physiology and proliferation were mentioned solely to illustrate the profound consequences which result from fixation of the adenylate cyclase G-protein in the GTP form. As stated in my article, which (if any) specific effector systems involve p21^{ras} in signal transduction remains to be established.

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The gyroscope test of relativity

SIR — Your account of the "relativity gyroscope experiment"¹ does not go beyond that of Schiff², who proposed that two effects, the geodetic (de Sitter or spin-orbit) and motional (Lense-Thirring or spin-spin) precessions, could be measured separately by the use of at least two gyros in free fall in a perfect polar orbit. An accuracy of 0.3 milliarc-seconds per year is the desired goal. However, there are many other contributions which are well above 0.3 marc s yr⁻¹ (for a review see ref. 3).

The goal of the experimentalists is to make a sphere of fused quartz accurate to one part in 10⁷, but even a perfect sphere is distorted when set spinning, acquiring a quadrupole moment, which for an altitude of 500 miles, will contribute more than 0.3 marc s yr⁻¹ to the gyro drift rate if the spin axis is more than 20 minutes of arc away from either the orbit plane or the perpendicular to the orbit plane⁴.

Second, the Earth's quadrupole moment will contribute an amount of 4 marc s yr⁻¹, more than ten times the desired accuracy, and, more subtly, distorts the satellite orbit so that, in averaging over a polar orbit, one obtains an additional "indirect contribution" of 1.33 marc s yr⁻¹.

Third, the contribution due to the Sun will give rise to a geodetic precession of the gyro (due to interaction of the spin of the gyro with its orbital angular momentum about the Sun), with the relatively

large value of 19.2 marc s yr⁻¹, while the deflection of light from the reference star (Rigel in Orion) can cause an apparent drift of the gyroscope of up to 14.4 marc s yr⁻¹.

The relativity gyro experiment test of general relativity is thus more complex than originally envisaged. Hence, there is a need for other tests of the geodetic and motional precessions, particularly the latter. A promising suggestion in this direction is the proposal of Scully and co-workers^{5,6} to use a ring laser interferometer to test both the geodetic and the motional precessions, which has the attraction that it can be carried out on Earth, while another proposal is to use a Foucault pendulum at the South Pole⁷. Also, the binary pulsar PSR1913+16 may provide a test of the spin-orbit precession and our calculations⁸ predict a precession rate for the pulsar spin axis of 1.23° yr⁻¹, based on the data of Taylor and Weisberg⁹.

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Mouse Igx sequence elements in *Drosophila*

SIR — Two sequence elements may be needed for correct transcription of the mouse immunoglobulin α gene. They are: TNATTTGCAT (dc), its inverted and complementary ATGCAAATNA (cd), upstream of the H-chain gene and TGCA⁶CTGTGNCCAG (pd)¹. The same sequences (8 out of 10 base pairs) ATTTGCAT and ATGCAAAT have been found by Parslow *et al.* independently². Sequences related to the above elements were found upstream of all human and mouse α and λ variable region genes and within the mouse heavy-chain enhancer in a homology of 50–100%. The same elements are present in high homology in chicken ovalbumin gene, sea urchin histone gene cluster and in other genes¹. I report here that the elements dc and cd are present upstream of the homoeotic gene *ftz*³ of *Drosophila*.

The element AGGCAAATAC is found upstream the coding region of *ftz* (first

nucleotide position -794)³. This has 78% homology with the cd element ATGCAAATNA. The sequence ATGTTTGCAT is also found upstream of the coding region of *ftz* (first nucleotide -854)³ and shares 78% homology with the dc element TNATTTGCAT. Other sequence elements can also be found upstream or within the *ftz* gene with less homology with the dc, cd or pd 50–67%, but this does not seem to be significant since dc, cd or pd can be found in many genes when homology less than 70% is considered (F. G. Falkner and H. G. Zachau, personal communication).

The dc and cd elements are highly conserved among many genes. Their location within the immunoglobulin genes suggests functional relation¹. The homoeotic gene *ftz* is associated with *Drosophila* development and contains a highly conserved region, the homoeo box, which has been found in other homoeotic genes — *Antp* and *Urb* of *Drosophila* as well as in a wide spectrum of animals. The relationship of conserved sequences needed for transcription to upstream sequences needed for segmentation during development is unknown. It is, of course, possible that the homologies might be chance events, and more information is needed before evolutionary or functional relationships are considered.

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FAULKNER AND ZACHAU REPLY — We agree that the occurrence of dc-related elements upstream of genes deserves attention. We quote some examples for that in our previous paper¹ and others in a forthcoming one (F. G. F., E. Neumann and H. G. Z., manuscript in preparation). At this point we would like to mention that a sequence fully homologous to dc was found upstream of all or most histone H2B genes², a point which we had missed previously. In general, we searched sequence libraries for dc at the 89% homology level since at lower levels too many sequences are picked up. But also dc (and pd) related sequences with lesser homology may be interesting if they can be correlated with putative regulatory functions. One example of this which we recently encountered is the finding of the dc and cd related sequences in the regulatory regions upstream of catabolite-sensitive genes of *Escherichia coli*^{3,4}.

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Inositol trisphosphate, a novel second messenger in cellular signal transduction

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There has recently been rapid progress in understanding receptors that generate intracellular signals from inositol lipids. One of these lipids, phosphatidylinositol 4,5-bisphosphate, is hydrolysed to diacylglycerol and inositol trisphosphate as part of a signal transduction mechanism for controlling a variety of cellular processes including secretion, metabolism, phototransduction and cell proliferation. Diacylglycerol operates within the plane of the membrane to activate protein kinase C, whereas inositol trisphosphate is released into the cytoplasm to function as a second messenger for mobilizing intracellular calcium.

THE behaviour of all cells from one instant to another is governed by signalling systems that translate external information into a limited repertoire of internal signals, second messengers. Receptors on the cell surface function as molecular antennae, detecting external information (for example, hormones, growth factors, neurotransmitters or light) which is then transduced and amplified into the second messengers which control many cellular processes such as metabolism, secretion, contraction, phototransduction and cell growth. Interest in this field of signal transduction is growing for several reasons, but perhaps not least because an imbalance of second messengers may be responsible for normal cells becoming cancerous.

The signalling system using cyclic AMP as the second messenger may be the best known. Until recently, we knew much less about the nature of the second messengers used by another major signalling pathway that utilizes the inositol lipids as part of a transduction mechanism. Stimulated inositol metabolism was discovered by Hokin and Hokin¹, who showed that the incorporation of ³²P into phospholipids in pancreas was stimulated by acetylcholine. It later became clear that a stimulated catabolism of inositol lipids occurs in many different cells in response to a wide range of external signals²⁻⁴. Durell and co-workers⁵ suggested that this stimulated metabolism may be a part of receptor function, while Michell subsequently proposed that inositol metabolism is concerned with the mode of action of those agonists which raise intracellular calcium⁶. Although this hypothesis is not universally accepted in its entirety^{6,7}, the many coincidences between calcium-mobilizing agonists and stimulated inositol turnover⁸, and many negative 'controls' (that is, receptors which do not mobilize calcium do not stimulate inositol catabolism) has led to a widespread acknowledgement that calcium and inositides are closely linked. It has also become clear that the mobilization of calcium from intracellular stores is an important source of activator calcium following stimulation of many different cells, including oocytes⁹, Swiss 3T3 cells¹⁰, pancreas¹¹, parotid¹², GH₃ pituitary cells¹³, blood platelets¹⁴, insulin-secreting β -cells¹⁵, liver¹⁶ and smooth muscle¹⁷. Thus, there has been a missing link not only between calcium and inositides, but also between the plasma membrane (where the relevant receptors lie) and the internal stores of calcium. Our hypothesis is that inositol trisphosphate (InsP₃) is that missing link.

Formation of inositol trisphosphate

The major metabolic pathways responsible for the formation and degradation of InsP₃ are illustrated in Figs 1 and 2 (for reviews see refs 3, 18). Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) is one of the inositol lipids located in the inner leaflet of the plasma membrane (Fig. 1), and is formed by a

two-stage phosphorylation of phosphatidylinositol, which is first phosphorylated at the 4-position of its inositol head group by a specific kinase to form phosphatidylinositol 4-phosphate (PtdIns(4)P); this is in turn further phosphorylated at the 5-position to give PtdIns(4,5)P₂, which is the immediate precursor used by the receptor mechanisms. As well as the two kinases responsible for the stepwise phosphorylation of phosphatidylinositol to PtdIns(4,5)P₂ (Fig. 1), there are corresponding phosphomonoesterases which convert PtdIns(4,5)P₂ back to phosphatidylinositol (Fig. 2) These kinases and phos-

Table 1 Summary of the tissues which respond to external stimuli by a change in the hydrolysis of PtdIns(4,5)P₂

Tissue	Stimulus
Rabbit iris smooth muscle	Acetylcholine ^{20,21*}
Brain	Acetylcholine ^{50*}
Parotid	Acetylcholine ^{50*,105*,106*} , substance p ^{50*}
Pancreas	Acetylcholine ^{72*} , caerulein ^{72*}
Avian salt gland	Acetylcholine ¹²⁰
Liver	Adrenaline ⁷¹ , ATP ⁷¹ , vasopressin ^{22,28*,30,58*} , angiotensin ⁷¹ , platelet activation factor ¹²¹
Blowfly salivary gland	Serotonin ^{23*,24*,122*}
Blood platelets	ADP ¹²³ , thrombin ^{124*,125} , platelet activating factor ¹²⁶
Sympathetic ganglion	Vasopressin ^{127*}
GH ₃ pituitary tumour cells	Thyrotropin-releasing hormone ^{25*,26*,27*,29*}
Adrenal cortex	Angiotensin ⁵⁴
Pancreatic islets	Glucose ¹²⁸
Neutrophils	f-Methionyl-leucyl-phenylalanine ^{129,130}
HL-60 leukaemic cells	f-Methionyl-leucyl-phenylalanine ^{131*}
Leukocytes	Pseudomonas leukocidin ¹³²
T-lymphoblastoid cell	Phytohaemagglutinin ¹³³
Leukaemic basophils	Antigen ^{134*}
Swiss 3T3 cells	PDGF ^{63*,88*} , bombesin ^{88*} , vasopressin ^{88*}
Neuroblastoma-glioma hybrid NG108-15	Bradykinin ¹³⁵
Photoreceptors	Photons ^{42*,43}
Sea urchin eggs	Spermatozoa ⁸⁵

PtdIns(4,5)P₂ hydrolysis was usually measured by following changes in the distribution of ³H-inositol or ³²P.

* Papers describing increases in the formation of labelled inositol trisphosphate (likely to be a mixture of the two isomers¹⁹). For more extensive lists of tissues where inositol turnover in general (rather than PtdIns(4,5)P₂ in particular) is stimulated, see refs 2, 8, 22.

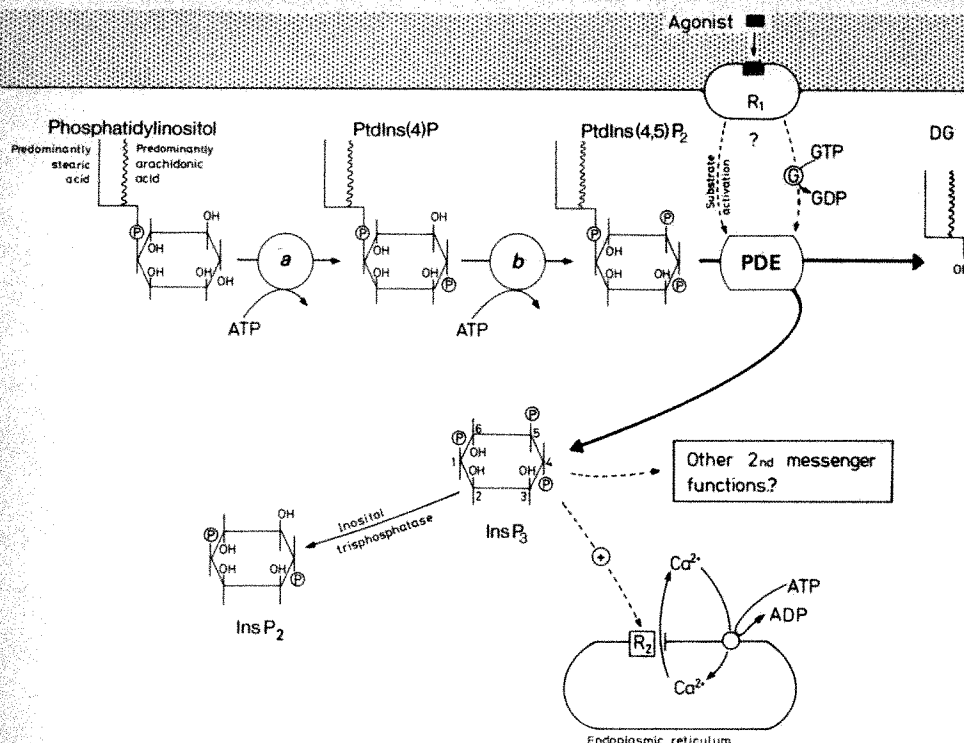


Fig. 1 The proposed role of inositol trisphosphate as an intracellular second messenger. Agonists bind to external receptors (R_1) to stimulate the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ by a phosphodiesterase (PDE) to form diacylglycerol (DG) and InsP_3 . The latter may have a number of second messenger functions one of which is to bind to a specific receptor (R_2) on the endoplasmic reticulum to release calcium. The action of InsP_3 is curtailed by an inositol trisphosphatase which removes a phosphate from the 5-position to form InsP_2 . The enzymatic pathways responsible for resynthesising phosphatidylinositol from diacylglycerol and InsP_3 are shown in Fig. 2. *a*, Phosphatidylinositol kinase; *b*, $\text{PtdIns}(4)\text{P}$ kinase; PDE, $\text{PtdIns}(4,5)\text{P}_2$ phosphodiesterase.

phosphoesterases constitute two linked phosphatidylinositol/ $\text{PtdIns}(4,5)\text{P}_2$ futile cycles whereby phosphates are constantly being added to and removed from the 4- and 5-positions of the inositol head group. Such futile cycles are metabolically expensive, which may explain why the operation of this signal transduction mechanism is rapidly curtailed by metabolic inhibitors⁴. Occupation of a receptor by an agonist diverts $\text{PtdIns}(4,5)\text{P}_2$ out of the futile cycle towards a phosphodiesterase (phospholipase C), by which it is cleaved into *sn*-1,2-diacylglycerol and InsP_2 (Figs 1, 2).

In passing, it should be noted that two naturally occurring isomers of inositol trisphosphate have been identified, D-*myo*-inositol 1,4,5-trisphosphate and D- or L-*myo*-inositol 1,3,4-trisphosphate¹⁹. In most of the studies summarized in Table 1, where increases in inositol trisphosphate following cell stimulation have been reported, the measurements probably include both isomers. Because the 1,3,4-isomer has only just been described and its function is unknown, we have concentrated our attention on the 1,4,5-isomer, which we abbreviate as InsP_3 .

Stimulated catabolism of one of the polyphosphoinositides was first detected by Durell *et al.*⁵, but other workers later documented in detail the phosphodiesterase cleavage of $\text{PtdIns}(4,5)\text{P}_2$ in iris smooth muscle to form InsP_3 (refs 20, 21). At first, however, this reaction was thought to be a consequence of the calcium signal in the stimulated tissue, rather than its cause. A crucial advance which changed our concept of inositide metabolism and its function, was the discovery that, in hepatocytes stimulated by vasopressin, decreases in $\text{PtdIns}(4,5)\text{P}_2$ precede changes in phosphatidylinositol and are largely independent of external calcium²². The primary receptor-stimulated event is probably the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ to yield *sn*-1,2-diacylglycerol (as does phosphodiesterase hydrolysis of any glycerophospholipid) and—uniquely to $\text{PtdIns}(4,5)\text{P}_2$ — InsP_3 as a water-soluble product^{3,4,22}. There are now many studies which support the notion that this hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ is a common response by many different kinds of cells to a wide variety of external stimuli (Table 1). The demonstration that the formation of InsP_3 precedes that of inositol monophosphate (the expected product of phosphatidylinositol hydrolysis) in *Calliphora* salivary glands^{23,24} and clonal GH₃ pituitary cells^{25–27} provides additional evidence that $\text{PtdIns}(4,5)\text{P}_2$ is the primary substrate for the receptor. Most importantly, InsP_3 formation in *Calliphora* salivary glands is

stimulated by serotonin with no apparent lag, whereas the onset of the calcium-dependent physiological response is delayed by at least 1 s. This led to the suggestion that InsP_3 is the second messenger for calcium mobilization^{23,24}. Increases in InsP_3 following cell stimulation also precede physiological responses in liver²⁸ and GH₃ cells²⁹, thus confirming that InsP_3 formation is fast enough for it to function as a second messenger.

One unsolved problem in the formation of InsP_3 and diacylglycerol is that of how receptors are coupled to the $\text{PtdIns}(4,5)\text{P}_2$ phosphodiesterase (Fig. 1). A recent report that vasopressin can stimulate the breakdown of $\text{PtdIns}(4,5)\text{P}_2$ in isolated membranes³⁰ may help to elucidate the transduction mechanism. Agonists may induce a conformational change in the receptor, which in turn perturbs the membrane sufficiently to make $\text{PtdIns}(4,5)\text{P}_2$ accessible to the phosphodiesterase³¹. $\text{PtdIns}(4,5)\text{P}_2$ phosphodiesterase may also be controlled by coupling of receptors to the enzyme through a GTP-binding protein as in the mechanism used to link receptors to adenylate cyclase^{32,33}.

The first indications that a GTP-binding protein may function in signal transduction for calcium-mobilizing receptors were reports that GTP reduces the affinity of noradrenaline for α_1 -receptors³⁴ and that of carbachol for muscarinic receptors³⁵. In adrenoreceptors, pertussis toxin reduces the modulatory effect of GTP on α_2 -receptors, which act by inhibiting adenylate cyclase, but has no effect on the calcium-mobilizing α_1 -receptors, suggesting that these two receptor mechanisms are regulated by different GTP-binding proteins³⁶.

A more direct link has emerged from studies on permeabilized blood platelets, where either GTP or its non-hydrolysable analogues GTP γ S or GppNHP enhances the calcium sensitivity of the secretory mechanism³⁷, apparently by stimulating the formation of diacylglycerol³⁸. The stimulatory effect of guanine nucleotides introduced into various intact cells can probably be reinterpreted on the basis that they stimulate the hydrolysis of inositol lipids³⁷. For example, stimulation of mast cell secretion by non-hydrolysable GTP analogues³² may result from stimulation of the normal receptor mechanism³⁷. Likewise, the ability of GTP γ S to excite *Limulus* photoreceptors may also depend on the activation of a GTP-binding protein^{39,40}. Such a protein has been identified in squid photoreceptors⁴¹, where light stimulates the turnover of $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ (refs 42, 43). Finally, the *ras* gene product, p 21, which is a membrane-bound

for the store are the mitochondria and the endoplasmic reticulum. These can be distinguished from each other by loading with calcium at different concentrations or by using various metabolic inhibitors. When calcium is buffered at 180 nM, permeabilized liver cells accumulate calcium in a non-mitochondrial pool which is sensitive to InsP_3 (ref. 58). Conversely, there is no release when cells are allowed to accumulate calcium in a mitochondrial pool^{58,59,66}. Inhibition of mitochondrial function by removing metabolic substrates or by adding inhibitors (antimycin, oligomycin, Ruthenium red) has no effect on the ability of InsP_3 to release calcium from the ATP-dependent vesicular pool^{57,59,62,67}. Thus, the InsP_3 -sensitive release site is probably the endoplasmic reticulum, not the mitochondria.

This conclusion is supported by cell fractionation experiments, which show that InsP_3 can release calcium from microsomes but not from isolated mitochondria^{65,69}. After further fractionation of pancreatic microsomes, InsP_3 -sensitive release is localized predominantly in those vesicles containing markers for the endoplasmic reticulum rather than the plasma membrane⁶⁹. Insulinoma microsomes do not respond to a second challenge with InsP_3 (ref. 65), which can probably be explained by vesicle heterogeneity⁶⁰. When InsP_3 is first added, calcium is chased out of the InsP_3 -sensitive vesicles only to be mopped up by those which are insensitive⁶⁰. This heterogeneity suggests that, within the intact cell, only a part of the endoplasmic reticulum, perhaps that lying close to the plasma membrane, may be sensitive to InsP_3 . Changes in intracellular calcium in response to agonists may, therefore, not be uniform, but may be localized in specific regions depending on the juxtaposition of surface receptors and intracellular calcium stores¹⁷. An analogous arrangement may occur in cardiac muscle, where calcium is thought to be taken up over the whole surface of the sarcoplasmic reticulum but to be released only from the terminal cisternae close to the T-tubules⁷⁰.

Previous studies with intact liver suggested that the hormone-dependent mobilization of calcium involves an amplification step, for more than 10 calcium ions are released for each molecule of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysed by the receptor mechanism⁷¹. One amplification step probably resides at the level of the endoplasmic reticulum; for example, using data from permeabilized liver cells⁵⁹, we calculate that each InsP_3 molecule releases at least 20 calcium ions. Just how this calcium is released from the endoplasmic reticulum is unknown, but current evidence favours the view that InsP_3 acts by stimulating calcium efflux (as shown in Fig. 1) rather than by inhibiting calcium uptake. When the latter is blocked completely, either by adding vanadate or by removing ATP, there is a gradual release of calcium which is very much slower than that obtained by adding InsP_3 (ref. 67). Furthermore, the calcium mobilizing effect of InsP_3 was greatly enhanced when calcium uptake was blocked. The ability of InsP_3 to release calcium from the endoplasmic reticulum was unaffected by nifedipine (5 μM), verapamil (10 μM), cobalt (100 μM), dantrolene (30 μM) or Ruthenium red (20 μM)⁶⁶.

The specificity of the putative InsP_3 receptor on the endoplasmic reticulum (R_2 in Fig. 1) has been analysed by testing a range of inositol phosphates and closely related compounds (Table 2). It is of particular interest that InsP_2 , formed when InsP_3 is hydrolysed⁴⁹ by inositol 1,4,5-trisphosphate 5-phosphatase (Fig. 1), has no effect. The InsP_3 -binding site on that enzyme is apparently distinct from the receptor on the endoplasmic reticulum, because 2,3-diphosphoglyceric acid, which blocks the trisphosphatase⁴⁹, has no effect on the ability of InsP_3 to release calcium⁶⁴. A common feature of the four inositol phosphates which can release calcium^{61,64} (Table 2) is that they all have the pair of vicinal phosphates at the 4- and 5-positions (Fig. 1). These two phosphates appear to be essential for full activity (ability to release calcium), whereas the remaining phosphate, at the 1-position, seems to enhance the affinity of InsP_3 for its receptor. Moving this phosphate from the 1- to the 2-position causes some reduction of affinity, and removing it altogether causes a dramatic reduction in the ability of the molecule to

Table 2 Specificity of InsP_3 -sensitive calcium mobilization

Inactive compounds	Concentrations tested (μM)
2,3-Diphosphoglycerate ⁶⁴	2,000
Fructose 1,6-bisphosphate ⁶³	10
<i>myo</i> -Inositol ^{57,58,63,65,66}	2.5–100
Inositol 1-phosphate ^{57,58,65}	5–10
Inositol 2-phosphate ^{63,66,67}	7.5–10
Inositol 1,2-cyclic phosphate ^{57,58,63}	5–10
Inositol 1,4-bisphosphate ^{57,58,63–65,67}	1–50

Active compounds	Concentration giving half-maximal release (μM)	Tissue
Inositol 1,4,5-trisphosphate	0.4	Rat pancreas acinar cells ⁵⁷
Inositol 1,4,5-trisphosphate	0.1	Rat hepatocytes ⁵⁹
Inositol 1,4,5-trisphosphate	0.2	Guinea pig hepatocytes ⁵⁸
Inositol 1,4,5-trisphosphate	0.3	Swiss 3T3 ^{63,64}
Inositol 1,4,5-trisphosphate	0.5	Rat insulinoma ⁶⁶
Inositol 1,4,5-trisphosphate	0.7	Porcine artery cells ⁶⁸
Inositol 1,4,5-trisphosphate	0.75	Human neutrophils ⁶⁷
Inositol 1,4,5-trisphosphate	0.8	Macrophages ¹³⁹
Inositol 1,4,5-trisphosphate	1.0	GH ₃ cells ⁶²
Inositol 1,4,5-trisphosphate	0.50	Rat liver microsomes ⁶⁰
Inositol 1,4,5-trisphosphate	3.0	Insulinoma microsomes ⁶⁵
Glycerophosphoinositol 4,5-bisphosphate	1.1	Swiss 3T3 ⁶⁴
Inositol 2,4,5-trisphosphate	1.1	Swiss 3T3 ⁶⁴
Inositol 2,4,5-trisphosphate	1.3	Guinea pig hepatocytes ⁶¹
Inositol 4,5-bisphosphate	20	Swiss 3T3 ⁶⁴
Inositol 4,5-bisphosphate	~50	Guinea pig hepatocytes ⁶¹

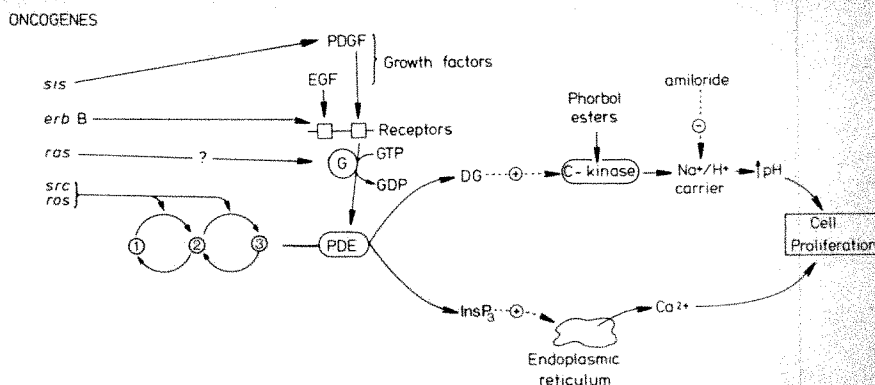
mobilize calcium (Table 2). The most effective molecule tested so far is one of the naturally occurring isomers¹⁹, with the three phosphates in a D-1,4,5-configuration.

The rate at which InsP_3 is formed after cell stimulation^{23,24,28,29} is fast enough for it to be the messenger causing calcium mobilization, but the concentration which it can reach in stimulated tissues is at present unknown. Rough estimates based on the $\text{PtdIns}(4,5)\text{P}_2$ content of tissues⁵⁷ and direct measurements of labelled InsP_3 (refs 28, 58, 72) give values in the micromolar range, which are within the levels active on permeabilized cells (Table 2). These measurements, however, all rely on isotopic techniques where the problems of pool equilibration and the existence of two isomers make accurate estimates impossible.

Phototransduction

Photoreceptors function as transducers by converting the energy of light into the electrical signals that convey visual information to the brain. A gap in our knowledge of phototransduction is the link between photon capture by rhodopsin, and the change in ion permeability that initiates electrical activity. The need for a second messenger in phototransduction is highlighted by the fact that a single photon can open approximately 1,000 sodium channels in *Limulus* photoreceptors³. That InsP_3 may be such a link in phototransduction^{42,73} is borne out by evidence that light causes a decrease^{42,43} in the amount of ³H-inositol in the precursor $\text{PtdIns}(4,5)\text{P}_2$, with a corresponding increase in the amount of labelled InsP_3 (ref. 42). Octopus photoreceptors possess a protein whose GTP binding and hydrolysis functions are sensitive to light⁴¹. As described earlier (Fig. 1), this GTP-binding protein may be responsible for coupling activated receptors (in this case bleached rhodopsin) to the $\text{PtdIns}(4,5)\text{P}_2$ phosphodiesterase which generates InsP_3 .

Fig. 4 The proposed role of diacylglycerol and InsP_3 as intracellular second messengers in regulating cell proliferation. Oncogenes appear to code for various aspects of the signal pathway such as growth factors (*sis*) and the kinases (*src* and *ros*) responsible for forming the substrate ($\text{PtdIns}(4,5)\text{P}_2$) used by the receptor mechanism. The *erb-B* gene codes for a truncated EGF receptor reported to stimulate phosphatidylinositol turnover by some workers^{87,137} but not by others¹³⁸. The *ras* gene product may function to couple receptors to underlying transduction mechanisms such as the $\text{PtdIns}(4,5)\text{P}_2$ phosphodiesterase (PDE). Diacylglycerol apparently acts by raising pH , whereas InsP_3 mobilizes intracellular calcium. 1, Phosphatidylinositol; 2, $\text{PtdIns}(4)\text{P}$; 3, $\text{PtdIns}(4,5)\text{P}_2$.



When injected into the ventral photoreceptors of *Limulus*, InsP_3 induces electrical events which are identical with those produced normally by light^{42,73}. InsP_3 stimulates an inward current, carried mainly by sodium ions, which has a reversal potential identical with that induced by light. As well as functioning as a second messenger in phototransduction, InsP_3 may also be responsible for the process of adaptation^{42,73}. Injection of InsP_3 into photoreceptors reduces their responsiveness to subsequent flashes of light. Conversely, a flash of light causes a reduction in the responses induced by repetitive injections of InsP_3 (ref. 42). As an increase in intracellular calcium has been implicated in adaptation⁷⁴, it is reasonable to suppose that some of the actions of InsP_3 in photoreceptors may be mediated by mobilizing calcium from internal stores. It remains to be seen whether or not InsP_3 is involved in vertebrate photoreceptors.

Cell growth and oncogenes

The discovery of InsP_3 as an intracellular second messenger may contribute to our understanding of those aspects of cell growth and development that depend on the mobilization of calcium from intracellular stores. It has been known for some time that growth factors cause changes in inositol lipid metabolism⁷⁵, but the link between these events at the plasma membrane and the onset of cell proliferation has not been understood. There is now much support for the proposal that both diacylglycerol and InsP_3 may function as second messengers controlling the two major ionic events implicated in cell proliferation (Fig. 4). Diacylglycerol acts within the plane of the membrane to stimulate protein kinase C⁵⁶, which is probably the site of action of the tumour-promoting phorbol esters⁷⁶. As the phorbol esters can activate the Na^+/H^+ exchange carrier^{77,78}, it has been proposed that diacylglycerol may act in a similar way to increase cytoplasmic pH (refs 4, 63). Such an increase in pH is a characteristic feature of the action of growth factors and is thought to have an important role in stimulating cell growth⁷⁹⁻⁸¹.

Calcium has long been implicated in cell proliferation^{82,83}. The increase in intracellular calcium which occurs when cells are fertilized⁹ or stimulated with growth factors^{10,84} may depend on the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ to form InsP_3 , which then releases calcium from intracellular stores. The large increase in polyphosphoinositide metabolism in sea urchin eggs following fertilization has been implicated in calcium mobilization⁸⁵. Direct evidence for the involvement of InsP_3 is provided by the observations that injection of InsP_3 (but not InsP_2) triggers some of the initial events of the fertilization process (elevation of the fertilization membrane) in sea urchin⁸⁶ and starfish oocytes (M.J.B. and L. Meijer, unpublished observations). The action of growth factors (platelet-derived growth factor (PDGF), bombesin and vasopressin) on Swiss 3T3 cells results in an increased formation of diacylglycerol⁸⁷ and InsP_3 (refs 63, 88). The latter is likely to function as a second messenger to mediate the calcium-mobilizing action of growth factors⁸⁹, as it can release calcium from permeabilized Swiss 3T3 cells^{63,64}. Thus, the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ induced by growth factors results in the activation of two signal pathways which may regulate

two key ionic events responsible for cell proliferation (Fig. 4). The increase in pH may be controlled through diacylglycerol, and the level of calcium by InsP_3 .

The enzymatic reactions associated with this bifurcating signal pathway based on the inositol lipids may represent the sites of action of certain oncogenes (Fig. 4)⁹⁰⁻⁹². The *sis* oncogene is responsible for producing PDGF^{93,94}, known to stimulate inositol lipid metabolism^{63,87}. The *erb-B* gene codes for a truncated EGF receptor⁹⁵. (EGF stimulates an immediate increase in cytoplasmic free calcium in fibroblasts^{10,84}.) Proteins encoded by the *src* and *ros* oncogenes may function as inositol lipid kinases^{90,91} to convert phosphatidylinositol into the $\text{PtdIns}(4,5)\text{P}_2$ used by the receptor mechanism (Fig. 4). It is reasonable to speculate that other oncogenes, whose functions are still unknown, may code for enzymes concerned with the remaining steps of this bifurcating signal pathway⁹². A particularly intriguing possibility is that the activated *ras* gene may transform cells by causing an uncontrolled stimulation of $\text{PtdIns}(4,5)\text{P}_2$ phosphodiesterase (Fig. 4). The normal *ras* gene can both bind and hydrolyse GTP, but on activation by a point mutation at codon 12, the resulting oncogenic protein can still bind GTP⁴⁵, although its ability to hydrolyse this nucleotide is severely impaired^{46,47}. If *ras* does play a part in this receptor mechanism, the loss of GTPase activity means that the oncogenic protein would continue to activate the formation of InsP_3 and diacylglycerol in an uncontrolled way independently of growth factors (Fig. 4).

Most of the oncogenes discovered so far have been identified through viral transformation or transfection assays and are likely to be dominant (drive the signal pathways forward by activating positive steps such as the formation of second messengers). It is conceivable, however, that there may be a set of recessive oncogenes which code for enzymes responsible for inhibitory aspects of the signal pathways such as second messenger removal. The potential targets for both dominant and recessive oncogenes are described elsewhere⁹².

Early development

Ionic currents flowing through oocytes may be responsible for segregating cytoplasmic components to establish an anterior-posterior axis during early development^{96,97}. A calcium-dependent chloride current entering the animal pole has been detected in *Xenopus* oocytes using an extracellular vibrating electrode⁹⁷. Muscarinic receptors, which are concentrated around the animal pole, respond to acetylcholine by inducing a membrane depolarization resulting from the opening of chloride channels^{98,99} themselves regulated by calcium, which is apparently released from intracellular stores by InsP_3 (ref. 100). Injection of eggs with InsP_3 results in an electrophysiological response similar to that normally induced by acetylcholine¹⁰⁰. Even in the absence of acetylcholine, eggs often display spontaneous fluctuations in chloride conductance⁹⁸ which probably account for the chloride current that enters the animal pole. These spontaneous fluctuations may result from the transient accumulation in the animal pole of the same 'channel gating substance'

normally released by acetylcholine⁹⁸ and which could well be InsP_3 . Therefore, a localized accumulation of calcium released by InsP_3 at the animal pole may be responsible for the large influx of chloride at this point. InsP_3 could thus have an important role in early development to establish an egg axis by segregating cytoplasmic components through localized calcium gradients and electric fields.

Calcium entry

In the initial seconds or minutes of cell activation by a number of agonists, the best demonstrated function of InsP_3 as a second messenger is the mobilization of calcium from intracellular stores, particularly the endoplasmic reticulum. If, however, a tissue is to maintain an elevated calcium level, some modification of plasma membrane calcium transport is necessary because of the limited size of the intracellular calcium stores. Whether or not InsP_3 is directly involved is not known, but elevated intracellular levels of calcium could be maintained by inhibition of the calcium pump on the plasma membrane^{101,102} by some mechanism regulated by changes in the level of $\text{PtdIns}(4,5)\text{P}_2$ (ref. 7). The $\text{PtdIns}(4,5)\text{P}_2$ used to generate InsP_3 is an integral part of the bilayer, where it may regulate a number of membrane properties in addition to controlling the calcium pump. The level of $\text{PtdIns}(4,5)\text{P}_2$ may regulate membrane fluidity¹⁰³ or the anchoring and transfer of proteins across the bilayer¹⁰⁴. So, as $\text{PtdIns}(4,5)\text{P}_2$ is consumed to generate second messengers, there may be localized perturbations in the bilayer which alter a number of membrane properties.

Inositol 1,3,4-trisphosphate

In several tissues, InsP_3 levels elevated in response to agonists are prolonged over many minutes^{28,58,105,106}, but a complicating factor in measuring InsP_3 levels is that the predominant inositol trisphosphate may not always be D-inositol(1,4,5)trisphosphate but, rather, D- or L-inositol(1,3,4)trisphosphate¹⁹. The origin and function of this is unknown, because the evidence for its existence comes from radiolabelling studies with ³H- or ¹⁴C-labelled inositol, its abundance in mass terms compared with the 1,4,5-isomer is also unknown. The occurrence of inositol(1,3,4)trisphosphate is therefore a further complication in assessing the rate and extent of InsP_3 accumulation. However, there is also a possibility that inositol(1,3,4)trisphosphate has messenger functions of its own and represents an entirely new facet of inositol function¹⁹. For example, hyperstimulation of the parotid via the cholinergic parasympathetic pathway, which results in a large accumulation of inositol(1,3,4)trisphosphate¹⁹, also stimulates DNA synthesis¹⁰⁷. It is also significant that treatment of cells with 5–20 mM lithium, which enhances the formation of inositol trisphosphate^{27,28,72} (much of which is likely to be the 1,3,4-isomer), is a potent growth stimulus in lymphocytes¹⁰⁸ and mammary¹⁰⁹ and kidney epithelial cells¹¹⁰. Although the 1,4,5-isomer seems to have more immediate effects, for example, calcium mobilization, the 1,3,4-isomer may contribute to longer-term responses, such as the onset of proliferation.

Second-messenger interactions

An important aspect of the bifurcating receptor mechanism which utilizes InsP_3 and diacylglycerol as second messengers is that the two limbs of the signal pathway may sometimes interact with each other synergistically⁵⁶, although an inhibitory effect of protein kinase C on calcium transport has also been reported¹¹¹. One explanation for the synergistic interactions is that the diacylglycerol-sensitive pathway may alter the sensitivity of the calcium pathway. For example, activation of protein kinase C by a phorbol ester results in a large increase in the calcium sensitivity of secretion in blood platelets¹¹² and adrenal medulla¹¹³. Some cellular processes, such as shape change and secretion in blood platelets, can be activated through the C-kinase pathway without a change in the resting level of calcium¹¹⁴, but a more effective stimulus is provided when both pathways act in concert^{56,112,114,115}. Another important interac-

tion is the finding that stimulation of the protein kinase C pathway can greatly enhance the formation of $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ (refs 116, 117). Diacylglycerol could thus function as a positive feedback signal to increase the formation of the $\text{PtdIns}(4,5)\text{P}_2$ required by the receptor mechanism^{116,117}.

Interactions between the cyclic nucleotide and inositol pathways are a relatively unexplored but potentially exciting area. In platelets, for example, cyclic AMP has a marked inhibitory effect on inositol lipid breakdown⁵⁶ (generally interpreted as an inhibition of the phosphodiesterase, but equally likely to be an effect on the kinases or phosphomonoesterases which control $\text{PtdIns}(4,5)\text{P}_2$ levels (Fig. 2)). Reciprocal effects of phorbol esters (presumably acting as diacylglycerol analogues) on the sensitivity of receptors which activate adenylate cyclase have also been reported^{118,119}.

The identification of InsP_3 as a second messenger raises far more questions than it answers. It is clear, however, that the bifurcating signal transduction system of inositol metabolism is used by a wide variety of agonists as a uniquely flexible signalling system. A key role for InsP_3 is to mobilize calcium, but its overall metabolic properties place it in a central position to carry out additional second messenger functions. The extent of its influence is evident from the growing list of tissues in which InsP_3 is a second messenger.

Note added in proof: In contrast to the lack of any effect of pertussis toxin on renal calcium-mobilizing receptors⁵⁰, this toxin inhibits GTP-dependent histamine release in mast cells¹⁴⁰.

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ARTICLES

Water column dissolution of aragonite in the Pacific Ocean

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Pelagic pteropods are an important component of the oceanic CO₂ system. Their shells appear to be a principal source of excess alkalinity in the upper water column of the North Pacific Ocean. Dissolution of aragonite must therefore be taken account of in generating models of the oceanic CO₂ system and is also an important cause of sample loss from long-term sediment traps deployed in undersaturated seawater.

MARINE aragonitic detritus is an important component of the oceanic CO₂ system and may constitute worldwide about one half the calcium carbonate (CaCO₃) detritus settling to the ocean floor¹; in some areas of the Pacific Ocean it appears to constitute substantially more than half the total CaCO₃ flux in the upper ocean². Aragonite dissolution provides a means of

explaining alkalinity anomalies in the upper ocean^{2,3} and, in addition, a means of balancing the oceanic CaCO₃ mass budget^{1,4}. Aragonite may be especially important in the short-term buffering of fossil fuel CO₂ injected into the ocean⁵.

The importance of aragonite in the oceans is largely attributable to its rapid dissolution kinetics compared with calcite.

Table 1 Aragonite dissolution rates

$\Omega_{\text{calcite}}^*$	Keir†	Morse <i>et al.</i> †	Eq. (8)†	Eq. (7)‡
1.50	0.09	0.36	0.30	0.46
1.30	0.9	1.9	1.7	1.6
1.15	3.0	4.2	4.2	3.5
1.00	7.5	8.1	8.2	6.6
0.85	16	14	15	12
0.75	25	18	20	16
0.65	38	25 (34)§	27	22
0.55	56	33 (65)§	36	30

Rates (% d⁻¹) predicted by Morse *et al.*⁶, Keir⁷ and this work as a function of saturation state at 5 °C, 34.03‰ salinity and pressures of 60–760 atm. In this analysis we have used the properties of a seawater sample collected at 26° N, 165° E in the North Pacific Ocean.

* Ω_{calcite} calculated using the equations and procedures in Millero¹¹.

† $\rho = 1.78$.

‡ $\rho = 2.05$.

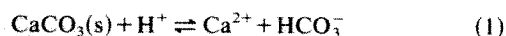
§ The higher dissolution rates shown in parentheses calculated using equation (11).

Consequently, these kinetics have received considerable attention recently. Substantial differences exist between results from careful laboratory investigations^{6,7} and *in situ* analyses^{8,9}; *in situ* aragonite dissolution rate determinations are more than an order of magnitude smaller than predictions based on laboratory analyses. These differences are a major concern in assessing the behaviour of aragonite in the oceans. We have performed a large number of dissolution rate determinations at sea, using freshly collected aragonitic particulates in an attempt to resolve the discrepancy. Our methods are only a small step removed from *in situ* analyses and incorporate advantageous features of laboratory methodology.

Analytical techniques

Our analyses were conducted aboard the RV *Discoverer*, along 165° E in the North Pacific Ocean (station locations and hydrographic data provided in Feely *et al.*¹⁰). Our pteropod samples were collected using free-drifting sediment traps at depths between 100 and 2,200 m. Use of large area (0.66 m²) traps allowed short (~24 h) collection periods and collection of intact pteropod shells with surface chemistries presumably quite similar to those of pteropods settling through the water column. The seawater used in our dissolution analyses was collected at the sediment trap stations along our transect². These samples were generally collected near 2,000 m and were characterized with respect to total CO₂, carbonate alkalinity and pH, as described previously¹⁰.

Our experiments were conducted by examining the dissolution of intact pteropod shells devoid of internal organic matter. In general, individual pteropods were used in each of our 80 experiments. Pteropods were housed in glass-plastic sample vials inside thermostated high-pressure vessels and were gently oscillated at 7 cycles min⁻¹ for the duration of each experiment. Each dissolution experiment was conducted at 5 °C and at pressures near 375 atm; extent of dissolution was monitored by measuring the seawater pH in the sample vials before and after each experiment. The pH changes induced by CaCO₃(s) dissolution



were generally quite small (0.03 ≤ pH ≤ 0.20). In order to maximize the signal-to-noise ratio, our seawater sample volumes were small (~9 ml) and pH was measured using electrodes (Ross-type reference—Orion Research) substantially free of drift in the course of thermal cycling. The total CaCO₃ dissolved in each experiment was determined using the initial alkalinity, pH and total CO₂ characteristics of our seawater samples¹⁰ and by calculating the alkalinity change (twice the change in total CO₂)

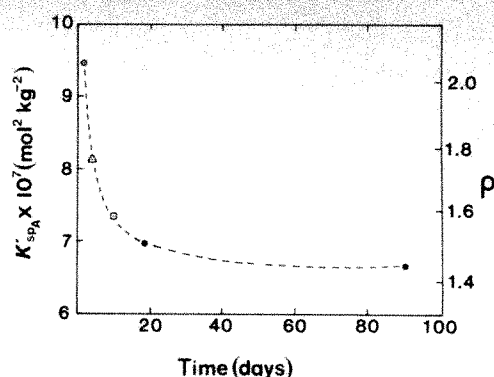


Fig. 1 The solubility behaviour of aragonite obtained in various investigations as a function of equilibration time¹⁸. The parameter $\rho = K'_{\text{spA}}/K'_{\text{spC}}$ provides a direct comparison of K'_{spC} , the solubility product of calcite obtained by Ingle *et al.*²⁰ (35‰ salinity, 25 °C) and K'_{spA} , values (35‰ salinity, 25 °C) obtained by: ○, Plath¹⁶; △, Berner¹⁵; □, MacIntyre¹⁷; ●, Morse *et al.*¹⁸. K'_{spC} values obtained in seawater at 25 °C and 35‰ salinity^{14-18,26} agree to within 5%. Consequently ρ is not significantly altered by alternative choices of K'_{spC} . Whereas the minimum value of ρ is reasonably well established, upper-bound values of ρ , appropriate to natural aragonitic particulates undergoing dissolution, are not well defined.

necessary to produce the observed change in pH. The equations and formation constants used in our calculations follow Millero¹¹ and Feely *et al.*¹⁰. Subsequent measurements of sample (pteropod) masses on laboratory microbalances permitted the result of each experiment to be expressed in terms of a % weight loss.

A potential liability encountered in the use of fresh pteropod shells is occasional evidence of bacterial respiration during dissolution experiments. We found an effective remedy for this possibility in the addition of Hg(II) to the seawater samples. Our seawater media were 2×10^{-5} M in Hg(II). Hg(II) has a very substantial affinity for halide ions, so Hg²⁺ is not readily available for interaction with carbonate surfaces. HgCl₄²⁻ substantially dominates the speciation scheme of Hg(II) in seawater¹² and, in our media, free Hg²⁺ concentrations are ~10⁻¹⁹ M.

Detailed laboratory experiments^{6,7,13} have shown that carbonate dissolution rates in seawater generally can be well described by the following expression

$$R = k(1 - \Omega)^n \quad (2)$$

where R is the dissolution rate (% per day), k has the dimensions % per day, n is the reaction order and Ω is the degree of saturation calculated as

$$\Omega = \frac{m_{\text{Ca}} m_{\text{CO}_3}}{K'_{\text{spA}}} \quad (3)$$

where m_{Ca} and m_{CO_3} are total Ca²⁺ and CO₃²⁻ concentrations in mol kg⁻¹ seawater, and K'_{spA} is the temperature, pressure and salinity-dependent apparent solubility product of aragonite in seawater. The cumulative % dissolution in each of our experiments was modelled using the following integral form of equation (2):

$$D = \int_0^t R dt = k \int_0^t \left[1 - \frac{m_{\text{Ca}} m_{\text{CO}_3}}{K'_{\text{spA}}} \right]^n dt \quad (4)$$

Dissolution times in these experiments ranged between one hour and approximately one day. The integrations were performed numerically by dividing the total dissolution time, t , in each experiment, into a large number of segments. Our analyses indicated that dissolution segments ≤ 1 min produced identical model predictions.

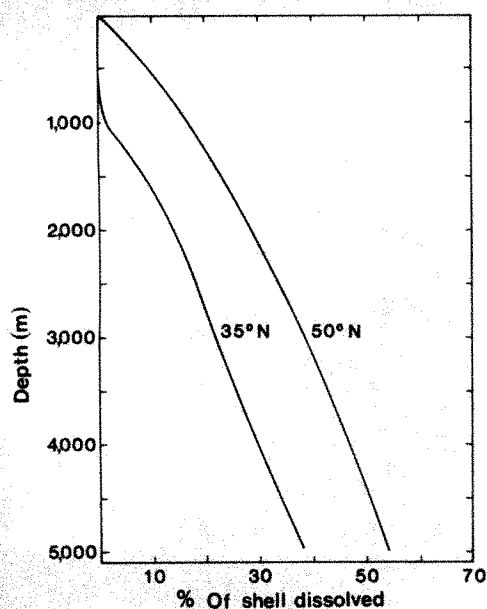


Fig. 2 The cumulative % dissolution of pteropods settling at a mid-range rate, 1.4 cm s^{-1} , at two of our station locations in the North Pacific Ocean. Significant dissolution begins at shallow depths at the northernmost station (50°N , 167°E). At 35°N , 165°E dissolution becomes appreciable below 1,000 m. As the influence of dissolution on settling rate is not considered, the cumulative dissolution shown here should be considered as a lower-bound estimate.

Equation (4) was applied here to each of our 80 dissolution experiments. In each analysis a set of parameters (k , K'_{spA} and n) was chosen and held constant. This process generated a value, $D(\text{predicted})$, for each of our experiments. $D(\text{predicted})$ was then compared with $D(\text{observed})$ and the difference was used to generate a residual, ϕ . The quality of a given set of parameters (k , K'_{spA} and n) was evaluated by examining the residual sum of squares generated by modelling the entire 80 experiment data set:

$$\text{residual sum of squares} = \sum_{i=1}^{80} \phi_i^2 \quad (5)$$

According to this process, the residual sum of squares was examined as a function of k , K'_{spA} and n . Our choices of K'_{spA} were constrained by the results obtained in a variety of direct aragonite solubility determinations¹⁴⁻¹⁸. An unusual feature of aragonite solubility relative to that of calcite is the long equilibrium time necessary for attainment of true equilibrium. Morse *et al.*¹⁸ demonstrated that where the aragonite solubility products obtained in various investigations are ordered with respect to equilibration time, a monotonic decrease is observed over a period of weeks (Fig. 1). This behaviour appears to reflect slow variation in the surface chemistry of aragonite¹⁹ and indicates that K'_{spA} appropriate to naturally occurring aragonitic particulates may assume values $\geq 6.65 \times 10^{-7} \text{ (mol}^2 \text{ kg}^{-2}\text{)}$. For convenience, K'_{spA} was expressed in terms of the apparent solubility product of calcite²⁰:

$$K'_{\text{spA}} = \rho K'_{\text{spC}} \quad (6)$$

Solubility analysis¹⁴ and partial molal volume data indicate that the effects of temperature and pressure on ρ are quite small compared with the changes in ρ attributable to variations in aragonite surface chemistry. K'_{spC} was calculated according to the methods of Millero¹¹ and Feely *et al.*¹⁰; values of ρ representative of the range of $K'_{\text{spA}}/K'_{\text{spC}}$ values obtained in detailed solubility analyses were used to calculate K'_{spA} . The values of ρ used here correspond to the lowest and highest values of ρ , 1.45¹⁸ and 2.05¹⁶ respectively, at 25°C and 35‰; and to the ratio,

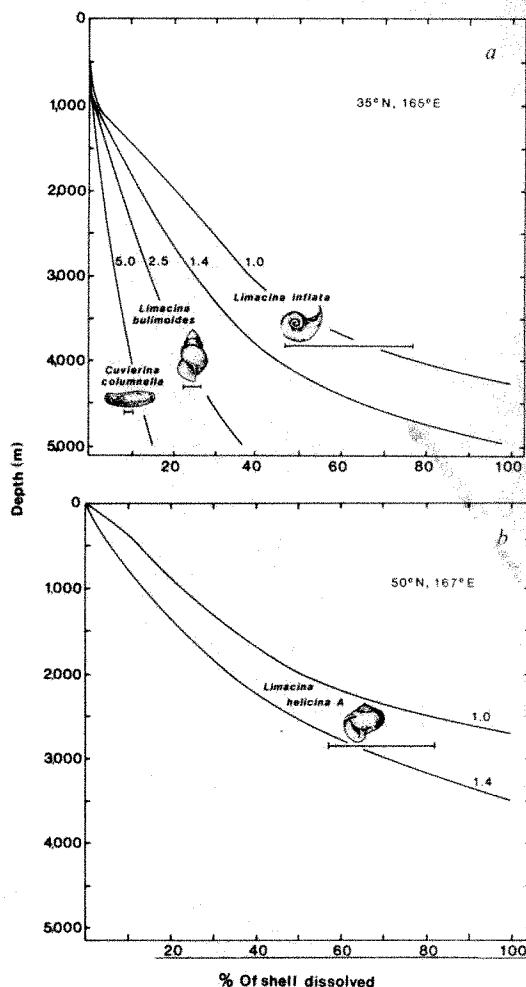


Fig. 3 *a, b*, The predicted cumulative % dissolution of pteropod shells in the North Pacific versus depth for representative initial settling velocities. The initial settling rates appropriate to each species are based on results obtained in our laboratory. The scales adjacent to each pteropod species represent a length of 1 mm. *b*, A single pteropod species was observed in the highly undersaturated waters at the northernmost station.

$\rho = 1.78$, appropriate to the use of Berner's K'_{spA} data¹⁵ in the dissolution analyses of Morse *et al.*¹⁶ and Keir⁷.

Aragonite dissolution rates

Examination of the residual sum of squares surfaces (RSS, k , n , ρ) generated by our data and the dissolution model, equation (4), produced the following results. First, the data could not be satisfactorily modelled using values of ρ as small as 1.45. Second, the best description of our results is provided by the equation

$$R = 125 \left[1 - \frac{m_{\text{Ca}} m_{\text{CO}_2}}{2.05 K'_{\text{spC}}} \right]^{4.1} \quad (7)$$

Third, the best fit model obtained with $\rho = 1.78$ produced reasonable descriptions of the data, only slightly inferior to the best fit results from equation (7)

$$R = 130 \left[1 - \frac{m_{\text{Ca}} m_{\text{CO}_2}}{1.78 K'_{\text{spC}}} \right]^{3.1} \quad (8)$$

Our dissolution rate results can be compared with the dissolution models of Morse *et al.*⁶ and Keir⁷. The dissolution rate equation of Keir⁷ is given by

$$R(\% \text{ per day}) = 318(1 - \Omega)^{4.2} \quad (9)$$

Morse *et al.*⁶ described their dissolution rate results using two

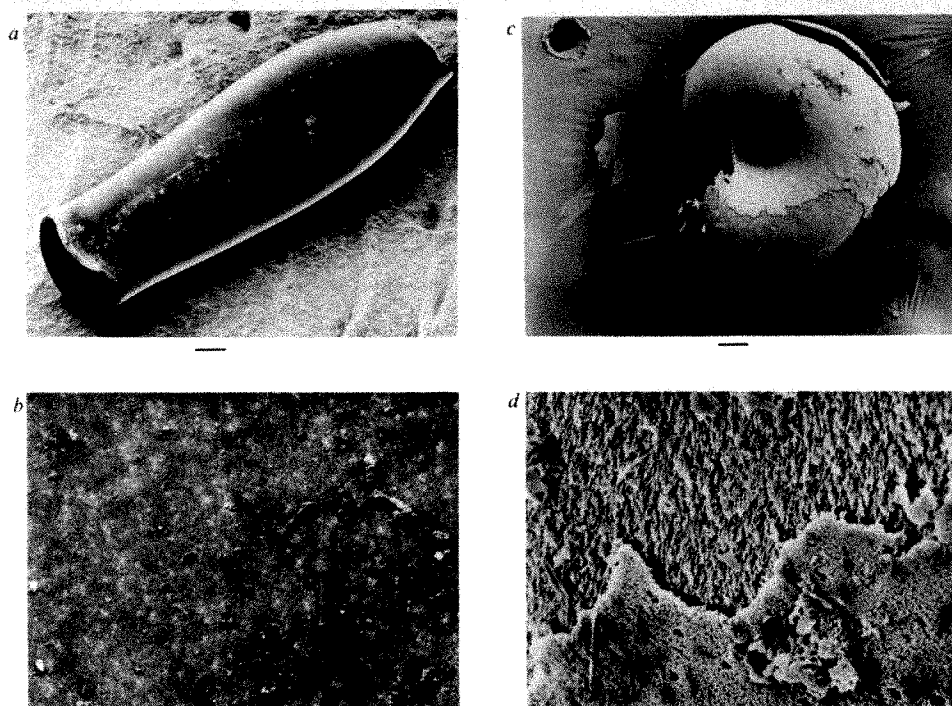


Fig. 4 The typical surface transformations accompanying pteropod dissolution. **a**, A smooth surface (*C. columnella*, scale bar 500 μm) typical of all large, rapidly settling species found in our sediment traps. **b**, Pteropods not exposed to undersaturated seawater exhibit smooth surfaces, even under high magnification (scale bar 10 μm). **c**, In contrast, the very thin-walled, slowly settling species, *L. inflata* (scale bar 100 μm) exhibits considerable surface etching. **d**, Under high magnification (scale bar 10 μm) this individual, taken from 2,100 m at 35° N, 165° E, shows the sharply contrasting surface characteristics typical of partially dissolved, slowly settling species.

equations. For $\Omega_A \geq 0.44$ they obtained the result

$$R(\% \text{ per day}) = 110(1 - \Omega)^{2.93} \quad (10)$$

and for higher degrees of undersaturation ($\Omega_A \leq 0.44$) they obtained

$$R(\% \text{ per day}) = 1,318(1 - \Omega)^{7.27} \quad (11)$$

Despite apparent differences in equations (7)–(11), the dissolution rate predictions of each are generally in good agreement. The dissolution rate predictions obtained here and by Morse *et al.*⁶ and Keir⁷ are compared (Table 1). In examining their predictions for the conditions of our experiments, we assumed a value of $\rho = 1.78$. This is consistent with their use of Berner's K'_{SPA} values.

The dissolution rates shown in Table 1 indicate that the determinations of Morse *et al.* and Keir provide good first-order predictions for the dissolution of aragonite in the oceans. This is important because previous comparisons have been either semiquantitative^{6,21} or in very poor agreement^{8,9} with laboratory results. In agreement with Morse *et al.*⁶ and Morse and Berner¹³, our results indicate that the *in situ* devices of Milliman⁸ and Honjo and Erez⁹ produced saturation states significantly exceeding the surrounding seawater.

Differences in the results obtained here and by Morse *et al.* and Keir can be attributed to a variety of factors. The enhanced dissolution rates observed by Keir are probably the result of using pteropod fragments rather than whole pteropods. The inner surfaces of intact pteropods have a somewhat limited availability for dissolution. Consequently, the experiments of Keir were conducted using materials of a somewhat larger surface area to mass ratio than the intact pteropods used here. The results of Morse *et al.*⁶ are in remarkable agreement with our results, except at high degrees of undersaturation. This indicates that our results are commensurate with equation (10) but not equation (11). This may indicate that, in the low temperature environment of the deep ocean, the change in reaction order observed at $\Omega_A = 0.44$ by Morse *et al.*⁶ occurs at much higher degrees of undersaturation. Finally, in agreement with

the solubility analyses of Morse *et al.*¹⁸, our results indicate that whereas lower bound K'_{SPA} values are reasonably well defined, the upper bound K'_{SPA} values appropriate to freshly collected pelagic aragonitic particulates are not well defined. $K'_{\text{SPA}}/K'_{\text{SPC}}$ ratios appropriate to freshly collected aragonite in undersaturated seawater appear to exceed significantly the ratio attained after long equilibration periods.

Water column dissolution of pteropods

The results given in equations (7)–(11) can be used to examine the cumulative dissolution of pteropods settling through the oceanic water column. Using the carbonate system characterizations (Ω_C versus depth) at two of our stations in the North Pacific¹⁰ we have calculated the cumulative % dissolution of pteropods settling at a mid-range rate²² of 1.4 cm s⁻¹. The results of this analysis at two locations along the cruise track are shown in Fig. 2.

At a mid-range settling rate, pteropods in transit to the floor of the Pacific Ocean will undergo substantial dissolution (Fig. 2). At high latitudes, dissolution rates are appreciable at shallow depths, whereas at low latitudes dissolution becomes appreciable at depths below 1 km. It is important to recognize that Fig. 2 was constructed without considering the decrease in settling velocity which accompanies mass loss. Therefore, we regard the extents of dissolution shown in Fig. 2 as lower bound estimates for the initial, 1.4 cm s⁻¹ settling velocity.

Analysis of the influence of mass loss on settling velocity is complicated by considerable variations in pteropod morphology. Nevertheless, a reasonable first-order assessment of the effect of mass loss on settling velocity can be constructed by assuming the following relationship:

$$v \equiv v_0 \left(\frac{m}{m_0} \right) \quad (12)$$

where v is pteropod settling velocity, v_0 is initial pteropod settling velocity, m_0 is initial pteropod shell mass, and m is pteropod shell mass corresponding to velocity v . Equation (12) is appropriate where substantial mass losses occur without

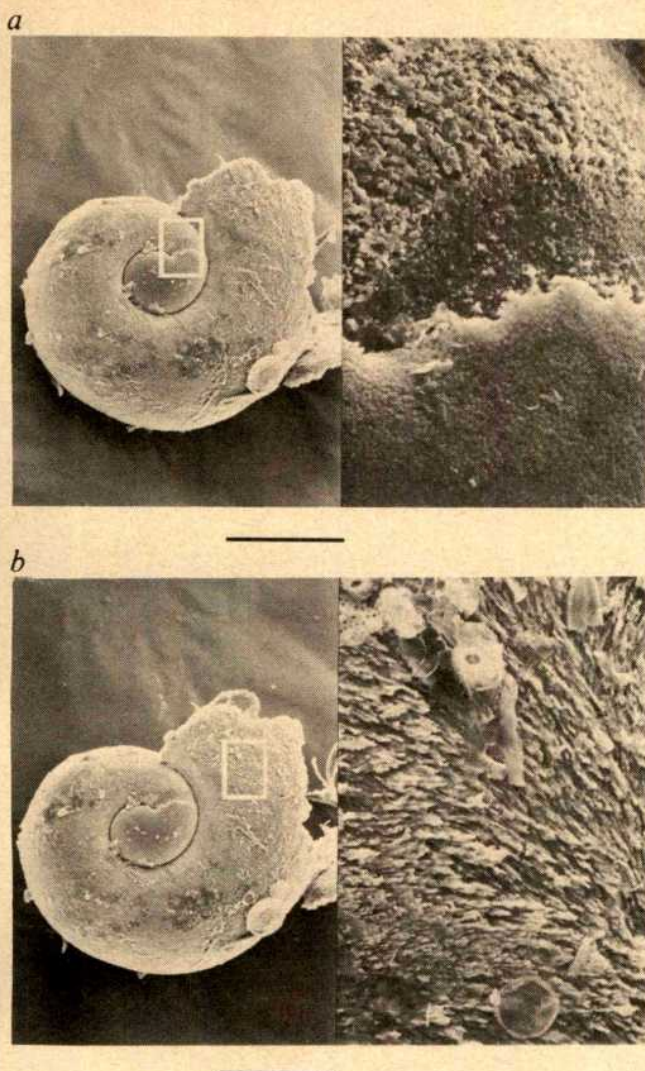


Fig. 5 Advanced stages of dissolution in smaller size class pteropods (immature *L. inflata*) found in the 2,100-m sediment traps at 35° N, 165° E. *a*, In this individual (scale bar 100 μ m) only a very small portion of the original smooth surface remains. *b*, The considerable surface roughening which occurs after extended exposure to highly undersaturated seawater.

appreciable changes in particle volume and, as such, is applicable to thin-walled shells until onset of fragmentation. Figure 3 shows the predicted dissolution behaviour of pteropods at several initial settling velocities, using equation (12) to account for reductions in settling velocity.

Discussion

Although our inability to deal with the consequences of shell fragmentation makes the predictions shown in Fig. 3 somewhat uncertain, it is clear that the influence of mass loss on settling rate can produce striking differences in the behaviour of pteropod shells in the water column. Shells settling at a mid-range rate are substantially dissolved before reaching the ocean floor. Shells settling with an initial velocity of 100 cm s^{-1} , appropriate to a major size class (juvenile *Limacina inflata*) observed in our mass flux investigations dissolve in the upper water column of the Pacific Ocean. It is important to note, however, that even in the corrosive waters found in the Pacific, large pteropods such as *Cuvierina columnella* undergo relatively minor dissolution before reaching the ocean floor. This explains the otherwise disconcerting observations that pteropods are found on the uppermost sediment of box cores²³ and as empty shells in the deep water column of the Pacific Ocean²⁴.

The results shown in Figs 2 and 3 have important implications in the interpretation of CaCO_3 flux measurements in the ocean.

Long-duration sediment trap deployments in undersaturated seawater will lead to large underestimations of aragonite fluxes. The aragonite fluxes observed by Betzer *et al.*², using short-duration sediment trap deployments, are quite substantial compared with previous results obtained through long-term collections. The flux measurements of Betzer *et al.* are lower bound estimates: large mobile pteropods which may have been able to swim into our sediment traps were not included in their estimates; because some accounting of large pteropod contributions to mass flux is certainly necessary, the significance of aragonite in the Pacific Ocean could exceed considerably the lower bound estimates. As an example of the potential significance of large pteropod species, the ratio of large-pteropod mass flux at 400-m depths to the small-pteropod mass flux reported in the conservative account of Betzer *et al.* is about 5:1.

Analysis of our sediment trap collections in the northwest Pacific Ocean indicates that pteropod shells having diameters <500 μm constitute at least 50% of the aragonite fluxes reported by Betzer *et al.*² for 100-m water depths. Shells in this size class, or smaller, should undergo extensive dissolution in the upper water column. Scanning electron microscopy of individuals in various pteropod size fractions provides direct evidence of extensive surface etching in the smaller size classes at relatively shallow depths. Figure 4 shows the contrasting dissolution features of the large rapid-settling pteropod species *C. columnella* and the small fragile species *L. inflata*. The smooth surface of *C. columnella*, even under considerable magnification (Fig. 4b), is typical of all large pteropod species collected in the deepest, 2,100 m, sediment traps. In contrast (Fig. 4c,d), *L. inflata* and other small pteropod species collected at 2,100-m depths exhibit extensive surface etching. At 2,100-m depths, slow-settling species have been in contact with undersaturated Pacific waters long enough (~ 1 –2 days) to have lost a considerable portion of their original integument. The smallest size fraction in our collections gave evidence of advanced dissolution: the *L. inflata* shell (Fig. 5) has retained only a small fraction of its original smooth surface; Fig. 5a shows the aragonite surface which initially results from loss of this integument; Fig. 5b shows the considerable roughening of the aragonite surface on further dissolution. The shell transformations exhibited by the small specimen in this figure should be characteristic of a shell settling at about 1 cm s^{-1} at 35° N (Fig. 3a). The abundant small size class aragonitic particulates observed in our studies should provide substantial contributions to the alkalinity of the upper water column of the Pacific Ocean. As such, we think that pteropods should be identified as the principal source of 'excess alkalinity' in the Pacific Ocean, described by Fiadeiro³.

According to Berner Berner and Honjo²⁵ and Betzer *et al.*², aragonite appears to be a major component of the marine CO_2 system. The results presented here indicate that aragonitic particulates should undergo substantial transformations in the upper water column of the Pacific Ocean. The relatively rapid dissolution kinetics observed in this study and previously^{6,7} indicate that detailed experimental assessments of aragonite fluxes in the Pacific Ocean should be conducted in a manner minimizing sample transformations subsequent to collection. This work, in conjunction with the analysis of Betzer *et al.*, provides substantial evidence that the appearance of alkalinity anomalies, in waters substantially supersaturated with respect to calcite³ can be attributed to water column dissolution of aragonitic pteropods.

We thank Captain Raymond Speer and the crew of the RV *Discoverer* for their effective assistance during our analyses; chief scientist Joel Cline for administering diverse research activities at sea, Chief Bosun Warren Sherril for sediment trap deployments and retrievals; Dr. John A. McGowan for discussions on the characteristics of pteropods in the Pacific Ocean; Anthony Grew for scanning electron microscope analyses and Thomas Kuba for computer programming assistance. This research was supported by a grant (NA80RAD00020) from the National Oceanic and Atmospheric Administration Air

Resources Laboratory and is Contribution No. 740 from Pacific Marine Environmental Laboratory. We acknowledge a Graduate Council Fellowship award to J.G.A. from the University of South Florida.

Received 16 August; accepted 27 September 1984.

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Characterization of the human factor VIII gene

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The complete 186,000 base-pair (bp) human factor VIII gene has been isolated and consists of 26 exons ranging in size from 69 to 3,106 bp and introns as large as 32.4 kilobases (kb). Nine kb of mRNA and protein-coding DNA has been sequenced and the mRNA termini have been mapped. The relationship between internal duplications in factor VIII and evolution of the gene is discussed.

HAEMOPHILIA has been known for millennia to be a male-specific, inherited disease¹. Haemophilia A afflicts 10-20 per 100,000 males; a relatively high proportion due to novel mutations of the factor VIII gene^{2,3}. The high frequency of haemophilia A compared with other autosomal clotting disorders results from the location of this gene on the X chromosome; one affected gene will thus produce the disease state in males. We report here the isolation and characterization of the 186,000 base pair (bp) region of the human X chromosome containing the complete factor VIII gene. A set of overlapping bacteriophage and cosmid clones was used to map and sequence the 26 exons containing the approximately 9,000 bp of coding sequence. Accompanying articles describe the expression of active human factor VIII from recombinant DNA clones⁴ and present the detailed characterization of the protein⁵. Our results provide a basis for studies of the molecular defects associated with haemophilia.

Factor VIII genomic clones

Initial factor VIII clones were recovered from a bacteriophage λ genomic library⁶ (λ 4X) containing DNA derived from an individual with 4X chromosomes (karyotyped 49,XXXXY). This library was screened with a unique 36-base oligonucleotide probe ('8.3'), synthesized to represent one codon choice of a sequenced tryptic peptide recovered from purified human factor VIII⁷. These initial clones contained overlapping segments spanning 28 kilobases (kb) of the human X chromosome. We subsequently expanded the set of genomic clones to contain 200 kb of the human genome encompassing the entire factor VIII gene. Both bacteriophage λ and cosmid clones were used in the process of 'genomic walking' (see legends to Figs. 1 and 2).

The restriction endonuclease map of the human factor VIII gene is shown in Fig. 1; the gene map was confirmed by the characterization of at least two independent clones for most of the 210-kb region and by numerous Southern blots of 49,XXXXY and normal DNA. No noticeable discrepancies between genomic DNA and the map derived from recombinant clones were found. In addition, low stringency blot experiments

have so far detected no closely related factor VIII genes or pseudogenes.

DNA sequence analysis

The DNA sequence of the entire mRNA coding portion (~9 kb) of the factor VIII gene has been determined. Exon-containing restriction fragments of genomic clones were identified by hybridization to cDNA and sequenced by dideoxy-chain termination procedures⁸. This DNA sequence analysis is distinct from that of cDNA clones derived from AL-7 cell RNA (see ref. 4). The genomic DNA sequence of exon boundaries (Fig. 3) and the promoter and polyadenylation regions (Fig. 4) are reported here. We found only two nucleotide differences between the genomic and cDNA sequences in the 8,860 nucleotides compared. Nucleotide 3,780 in cDNA is the G of a glutamic acid codon GAG, whereas in the cloned genomic DNA it is C, creating the conservative substitution of an aspartic acid residue. Nucleotide 8,728 in the 3' untranslated region is G in cDNA and A in genomic DNA. The surprising degree of similarity between genomic sequence derived from the 49,XXXXY cell DNA and cDNA sequence from AL-7 cell RNA indicates that the sequence data and the reverse transcription process are reliable and suggests also a lower level of nucleotide sequence polymorphism of this X-linked gene than has been found for some autosomal genes, an effect noted for other X chromosome loci⁹.

The boundaries of exons shown in Fig. 3 were established by restriction mapping and DNA sequence comparison of cDNA with genomic clones. In cases of nucleotide redundancy at either side of an intron, boundaries were chosen to be consistent with established consensus splice sites^{8,9}. All of the splice donor and acceptor sites conform to the GT...AG rule for nucleotides immediately flanking exon borders (Fig. 3). Further flanking sequences are in general agreement with favoured nucleotide frequencies noted in other compendia^{8,9}. The dinucleotide AG never occurs in the pyrimidine-rich region encompassing the final 15 nucleotides of introns; exons frequently begin with the sequence GT.

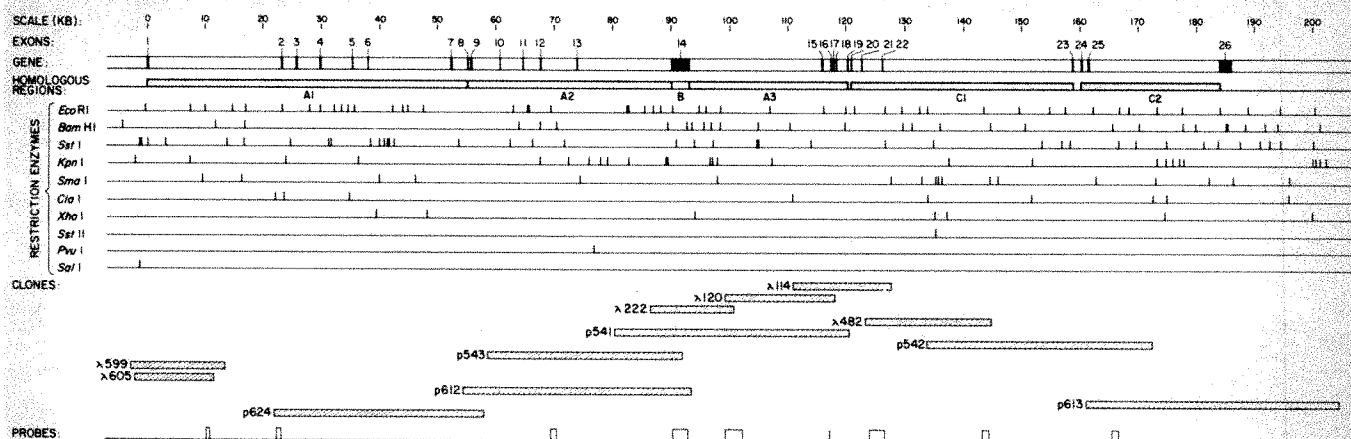


Fig. 1 Map of human factor VIII gene. The structure of the gene is schematically represented by an open bar; the 26 exons are filled-in areas drawn to scale. The direction of transcription is from left to right. The size scale in kb is drawn above the gene; the initiating ATG is positioned at nucleotide 1. Immediately below the gene are the extent of the triplicated 'A' domain, the unique 'B' domain, and the duplicated 'C' domain. The location of the recognition sites for the 10 restriction enzymes used to map the factor VIII gene are given in the next series of lines. Cross-hatched boxes represent the extent of human genomic DNA contained in each λ phage and cosmid clone. Other clones which encompass the same regions are not shown. No genomic clones span the 8.5 kb of intron located between clones λ 599 and p624. Other *EcoRI*, *BamHI* or *SstI* sites may lie in this region. The extent of this gap was determined by probing genomic Southern blots with single copy fragments isolated from the 3' end of λ 599 and the 5' end of p624. The bottom line shows the location of synthetic oligonucleotide or restriction fragment probes used in the genomic cloning, from left to right: the 2 probes used to map the uncloned region, three 5' walk probes, the original synthetic 36-mer probe '8.3'; three 3' walk probes.

Methods: λ and cosmid libraries were screened with random calf-thymus primed ^{32}P -labelled single copy genomic or cDNA probes²⁷. Nitrocellulose filters were hybridized overnight at 42 °C in 50% formamide, 0.1 g l⁻¹ salmon sperm DNA, 5 $\times$ SSC, 0.05 M sodium phosphate (pH 6.8), 5 $\times$ Denhardt's solution and 10% dextran sulphate²⁸. Filters were washed in 0.2 $\times$ SSC, 0.1 g l⁻¹ SDS at 60 °C for 1 h. Library filters were repeatedly rescreened with newly isolated walk probes so that extending clones were easily identified. To map restriction endonuclease cleavage sites, DNA from the clones was digested with restriction enzymes singly or in combinations and characterized by gel electrophoresis (followed by Southern blot hybridization in some cases). DNA fragments generated by *EcoRI* and *BamHI* digestion were subcloned into pUC plasmid vectors²⁹ for further propagation and analysis. Restriction mapping, DNA sequence analysis and blot hybridizations with the 8.3 probe determined the orientation of transcription of the gene. The genome walk was initiated by identifying single copy probe fragments near the ends of the initial 28 kb region. To find suitable fragments, digests of cloned DNA were blot hybridized with total ^{32}P -labelled human DNA under conditions where only DNA fragments containing sequences repeated more than about 50 times in the genome will hybridize^{30,31}. Candidate fragments were retested for repeated sequences by hybridization to 50,000 phage from the λ /4X library. To begin extension in the 5' direction, clone λ 222 was identified by a triplet of 1 kb *NdeI*/*BamHI* probe fragments isolated from λ 120 DNA. In the 3' direction, a single copy probe fragment of λ 114 failed to yield extending clones in screens of several λ libraries. Based on genomic blotting results, we then constructed a size selected³² *BclI* library of human 49,XXXXY DNA in the vector λ 1059³³. λ 482 is one of the extending clones so derived. Subsequent overlapping clones (p541, p542, p543, p612, p613 and p624) were derived by screening a recombinant cosmid library containing human DNA (see Fig. 2). The most 5' genomic clones, λ 599 and λ 605 were obtained by screening with cDNA-derived probes.

Fig. 2 Cosmid vector pGcos4. To facilitate genomic walking, we developed a new cosmid vector with the following features: (1) It can accommodate 45 kb inserts. (2) It confers resistance to tetracycline, rather than ampicillin. (3) The 641 bp *AvaI*/*PvuII* fragment of pBR322 has been removed to increase plasmid copy number and to delete sequences interfering with transformation of eukaryotic cells³⁴. (4) A methotrexate-resistant dihydrofolate reductase gene driven by a simian virus 40 (SV40) early promoter has been incorporated for the selection and propagation of clones in eukaryotic cells³⁵. (5) A synthetic DNA fragment containing a unique *BamHI* site flanked by pairs of *PvuI* and *EcoRI* sites has been inserted as the cloning site. The flanking *EcoRI* sites are useful for subcloning fragments and the *PvuI* sites allow excision of the entire insert in most cases, since this enzyme cuts eukaryotic DNA only about once per 135,000 bp. Construction: The scale is represented by dots within the circle numbered in kb from the zero point. The 403 base (b) annealed *HincII* fragment of λ c1857S7 (Bethesda Research Lab) containing the *cos* site was cloned in pBR322 from *AvaI* to *PvuII* to generate the plasmid pGcos1. Separately, the 1,624 b *PvuII* to *NaeI* fragment of pFR400³⁵ containing an SV40 origin and promoter, a methotrexate-resistant dihydrofolate reductase gene, and hepatitis B surface antigen polyadenylation sequences, was cloned into the pBR322 *AhaIII* site to generate the plasmid mp33dhfr. The following three fragments were ligated together to generate the cosmid vector pGcos3: 1,497 base (b) *SphI*-*NdeI* fragment of pGcos1, 2,163 b *NdeI*-*EcoRV* fragment of mp33dhfr and 376 b *EcoRV*-*SphI* fragment of pKT19. pKT19 is a derivative of pBR322 in which the *BamHI* site in the tetracycline resistance gene has been removed (provided by Herb Boyer). pGcos4 was generated by cloning the synthetic 20mer, 5' AATTCGATCGGATCCGATCG, in the *EcoRI* of pGcos3. Left and right arms of this vector were prepared by cleavage of two aliquots with *SstI* or *SalI*, followed by treatment with alkaline phosphatase, cleavage with *BamHI* and isolation of the large fragments²⁸. 49,XXXXY DNA isolated from the GM1202A cell line (NIGMS Human Genetic Mutant Cell Repository, Camden, New Jersey) was partially cleaved with five levels of *Sau3A1* and the 45 kb fragments isolated by sucrose density centrifugation²⁸. These fragments were ligated to the *BamHI* arms of pGcos4, packaged *in vitro* and used to infect *E. coli* HB101.

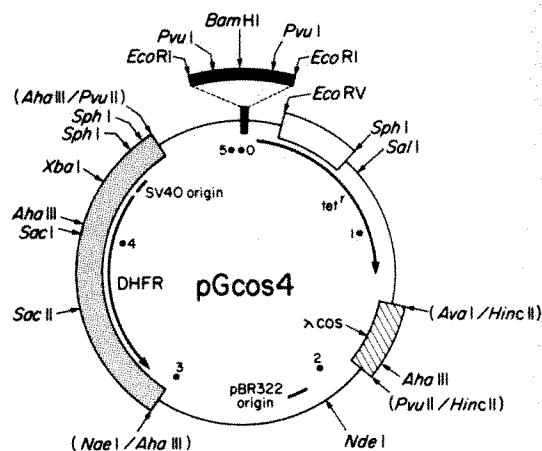


Fig. 5 Determination of the 5' mRNA start site by RNase protection. Autoradiograph of a 6% polyacrylamide, 7 M urea gel. Lanes 1 and 2, a known DNA sequencing ladder employed as size standards; lanes 3–11, results of RNase protection reactions.

Methods: Cell and tissue RNA was prepared³⁶. The 5' genomic *Sst*I fragment was ligated into pSP64 (a gift of P. Mellon and T. Maniatis) and linearized with *Pvu*II. The uniformly labelled ³²P-RNA probe was synthesized with 400 Ci mmole⁻¹ α -[³²P]CTP (Amersham) and SP6 RNA polymerase (NEN) followed by DNase treatment (DPRF, Worthington) as described^{12,13}. Sample RNAs were hybridized with 5 $\times$ 10⁵ c.p.m. of probe at 37°C and digested with 80 μ g ml⁻¹ RNase A and 4 μ g ml⁻¹ RNase T1 (Worthington) followed by extraction, electrophoresis and autoradiography. ³²P-labelled, anti-sense strand RNA probe was prepared by ligating the 1.2 kb *Sst*I genomic fragment containing exon 1 and 5' flanking sequences into pSP64¹² and transcribing the linearized plasmid with SP6 RNA polymerase^{12,13}. This labelled probe was hybridized with poly(A)⁺ RNA derived from various sources and digested with RNase A and RNase T1 before electrophoresis. Protected RNA was derived from: lane 3, AL-7 human hybridoma cells (10 μ g); 4, human liver sample 1 (10 μ g); 5, human liver sample 2 (10 μ g); 6, human Alexander cell line (10 μ g); 7, human Hep G2 cell line (10 μ g); 8, peripheral blood lymphocyte sample 1 (3 μ g); 9, peripheral blood lymphocyte sample 2 (3 μ g); 10, mouse T lymphoma cell line S49 (10 μ g); 11, no RNA control. An identical pair of protected fragments (arrow), measuring 185 and 187 bases from the *Sst*I site, appear only in RNA obtained from the two human liver samples and the AL-7 hybridoma cells. This positions the probable 5' mRNA start site at ~170 bases 5' of the initiation ATG as shown in Fig. 4. (As RNases A and T1 cannot cleave after an adenosine residue and the complementary strand is radiolabelled, no protected band was seen corresponding to a terminus at position -171 of the gene (Fig. 4), which is a T residue in the genomic sequence.)

the protected RNA is bounded by an intron instead of the true mRNA 5' start site.

The RNase protection experiments also show that liver is a site of synthesis of factor VIII. No factor VIII RNA was detected in the other tissues of the protection experiment shown in Fig. 5 nor by Northern blot analysis of RNA from various tissues and cell lines⁴. Liver is only one of several candidates for the site of factor VIII production^{14–16}.

Inspection of the genomic sequence of the 5' flanking region (Fig. 4a) revealed the sequence GATAAA beginning 30 bp 5' of the presumed 5' mRNA start site, similar to the canonical 'ATA' or 'Goldberg-Hogness' sequence, located about 25–35 bp before eukaryotic cap sites and apparently required for precise initiation of transcription by RNA polymerase II^{17,18}. We found no convincing candidate for the less frequently conserved 'CAT' sequence¹⁹ in the -70 bp region.

One additional ATG to nucleotide 5' to +1 is found in the 5' untranslated region at position -131 (Fig. 5). This region cannot serve as the translation start site for factor VIII because seven stop codons, distributed in all three possible reading frames, occur in the 128 nucleotides between this upstream ATG and position +1. Although only 5% of the previously compiled eukaryotic mRNAs contain an ATG 5' of the translation initiation site²⁰, the presence of an upstream ATG does not necessarily repress translational initiation at the proper start site when it is followed by an in-frame termination codon²¹.

The 3' untranslated region of the factor VIII gene contains 1,805 nucleotides (counting the TGA stop triplet and terminating before the first A of the poly(A) tail; Fig. 4b). The site of polyadenylation was inferred from cDNA clones and is preceded 19 bases by the common polyadenylation signal AATAAA²² as well as a second, less conserved recognition element CATTG²³.

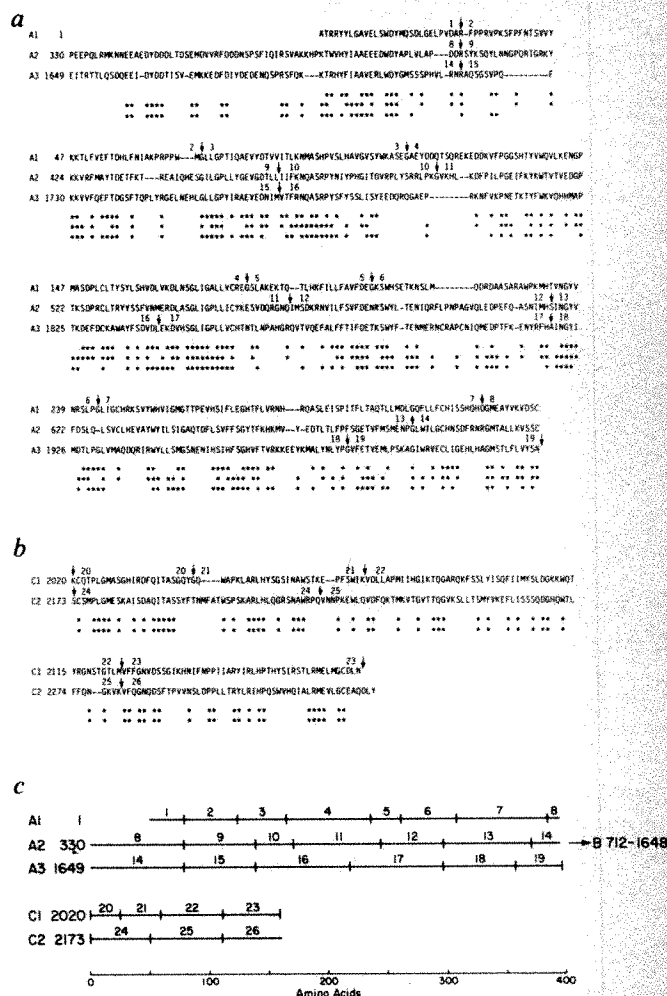
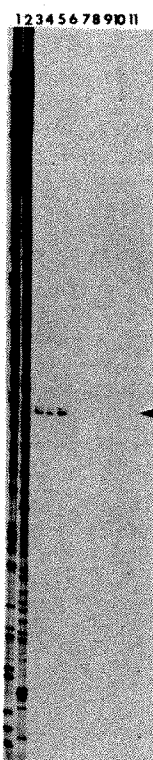


Fig. 6 Homologous factor VIII domains and intron boundaries. **a**, 'A' unit triplication showing the alignment of three amino acid sequences. The amino acid numbers precede each line. Arrows indicate the location of introns and are flanked by the appropriate exon numbers. *, Conserved amino acids. **b**, 'C' unit duplication. **c**, Representation of the A and C repeat units with the location of introns indicated by vertical lines. The exons are numbered.

Introns and protein domains

Searches for internal homologies in the factor VIII DNA sequence and the derived amino acid sequence revealed three types of domains: a triplicated 'A' unit of 330–380 amino acids, a unique 'B' unit of about 925 amino acids and a duplicated 'C' unit of about 160 amino acids. The order of the domains in the gene and protein is A1–A2–B–A3–C1–C2 (refs 4, 5; Fig. 1) (note that the exons constituting each domain are not clustered). The amino acid alignment of the A and C repeats (Fig. 6) demonstrates that the A units have ~30% pairwise amino acid homology (20% of the residues being conserved in all three domains), whereas the two C units have 37% homology^{4,5}.

If segmental DNA duplication has played a role in the evolution of the large factor VIII gene, one might expect a conservation of the intron boundaries within the A and C repeats of factor VIII. The alignment of most intron boundaries relative to the protein coding sequence is well fixed by the high degree of homology in the A and C repeats (Fig. 6). For the C duplication, intron boundaries occur precisely at the borders of the C1 and C2 repeats, as would be expected for a DNA duplication mechanism where the boundaries fall within the introns. Furthermore, the junction between A3 and C1 also contains an intron precisely at the boundary, again as expected for an intron joining mechanism. On the other hand, the A1–A2 and A2–A3 junctions are each contained on one exon (Fig. 6).

Within the A and C repeats only some of the intron boundaries

are conserved. In fact, each of the repeats contains a different number of exons. The precise alignment of some intron boundaries within the A and C repeats suggests that these introns were present in an ancestral precursor of the repeat units and duplicated in the course of the event that generated the repeat. The non-aligned introns appear to have arisen since the duplication, either by loss of one member of a duplicated intron or by the insertion of new introns after the duplication. In an analogous situation, α -feto protein and albumin each have a triplicated gene structure, but unlike factor VIII, all of the nine intron boundaries within the repeats for these two genes are conserved precisely²⁴⁻²⁵.

It is also interesting to speculate as to the origin of domain B in factor VIII. The 925 amino acids of this domain encompass nearly all of the 3.1 kb of exon 14, where the end of the A2 repeat (at the 5' end) and the start of the A3 repeat (at the 3' end) are also found. This suggests that during the course of evolution a processed gene may have been inserted into a short exon that contained the A2-A3 boundary. Insertion of a processed gene (derived from an mRNA) would also account for the anomalously large size of exon 14. The B domain connects the *M*_r 90,000 (made up of A1 and A2) and the *M*_r 80,000 (A3, C1, C2) fragments of the factor VIII protein. Proteolytic removal of the domain is associated with activation of the protein^{5,11}.

thus exon 14 is related to a physiological unit of the protein. Whether any other functional domains or subdomains of factor VIII correspond to any of the exons awaits further characterization of the molecule.

In conclusion, we report here the complete isolation and characterization of the human factor VIII gene. It is the largest gene yet characterized, encompassing nearly 0.1% (186 kb) of the human X chromosome. Transcription of such a large gene could take over three hours (based on a transcription rate of 15 nucleotides per second²⁶). Probes derived from the factor VIII gene will allow identification of restriction fragment length polymorphisms which can be used further to localize the gene on the X chromosome and lead to improved prenatal diagnosis of haemophilia. We anticipate that the knowledge of the gene structure and DNA sequence will be invaluable in the analysis of the molecular basis of haemophilia.

We thank John McGrath who characterized the λ 222 clone and Philip Hollingshead for laboratory assistance; Edward Tuddenham, David Martin, Ingrid Caras and Glenn Nedwin for providing tissue samples; Jeanne Arch and Alane Gray for preparation of the manuscript and figures. We thank David Goeddel, Christian Simonsen, Laurence Lasky and Dennis Kleid for helpful discussions and David Martin and Robert Swanson for their support.

Received 9 August; accepted 27 September 1984.

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Expression of active human factor VIII from recombinant DNA clones

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DNA clones encoding the complete 2,351 amino acid sequence for human factor VIII have been isolated and used to produce biologically active factor VIII in cultured mammalian cells. The recombinant protein corrects the clotting time of plasma from haemophiliacs and has many of the biochemical and immunological characteristics of serum-derived factor VIII.

A CRITICAL factor in the arrest of bleeding is the activation of the coagulation cascade, a system consisting of over a dozen interacting proteins present in plasma or released from cells^{1,2}. A small stimulus at the beginning of the cascade is catalytically amplified at each step such that the final outcome is the rapid conversion of the soluble protein fibrinogen into its insoluble

form, fibrin, which stabilizes the platelet plug. The lack or deficiency of any of the proteins involved in the coagulation cascade blocks the propagation of the initial stimulus. In the middle of the cascade is a step wherein factor IXa, in conjunction with factor VIII, converts factor X to the activated form, factor Xa. Factor VIII acts as a cofactor at this step, being required

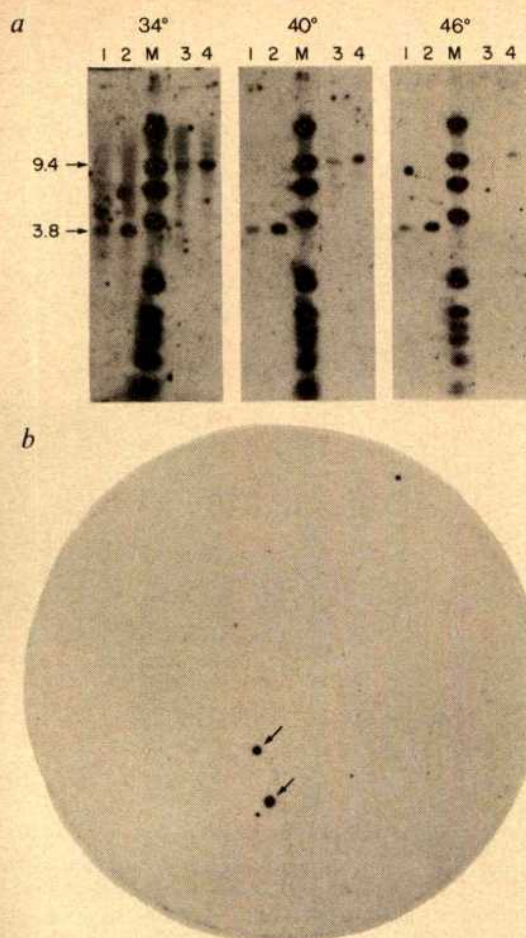


Fig. 1 Detection of the factor VIII gene with probe 8.3, 5'-CTTTTCAGGTCAACGTCGGAGAAATAAGCCCAAGC, a 36-base long probe synthesized based on the sequence of a 12 amino acid tryptic fragment of factor VIII, AWAYFSDVDLEK (ref. 5). **a**, Southern blots of 46,XY (1X, male) DNA and 49,XXXXY (4X) human DNA (isolated from cell line GM1202A, NIGMS Human Genetic Mutant Cell Repository, Camden, New Jersey) digested with *EcoRI* and *BamHI* were hybridized with ^{32} P end-labelled 8.3 probe in $6\times$ SSC, 50 mM sodium phosphate (pH 6.8), $5\times$ Denhardt's solution, 0.1 g l^{-1} boiled, sonicated salmon sperm DNA, 20% formamide and 10% dextran sulphate at 42°C . The three blots were washed in $1\times$ SSC, 0.1% SDS at the temperature indicated. Lane 1, *EcoRI* 1X; lane 2, *EcoRI* 4X; lane 3, *BamHI* 1X; lane 4, *BamHI* 4X; lane M, end-labelled λ HindIII and Φ X174 *HaeIII* digested marker fragments. **b**, One nitrocellulose filter from the screen of cloned 4X human DNA hybridized with probe 8.3. This library was constructed by partially digesting 4X DNA with *Sau3AI* and ligating the 15 kb size fraction into the vector λ Charon 30 (ref. 42). Arrows, the independent factor VIII positive clones. Hybridization and washing for the library screen was as described above for the Southern blots with a wash temperature of 37°C .

with calcium ions and phospholipid for the activity of factor IXa. The two most common haemophilic disorders are caused by the decreased function of either factor VIII (haemophilia A or classic haemophilia; 80% of all cases) or factor IXa (haemophilia B or Christmas disease). The clinical manifestation in both types of disorders is the same: a lack of sufficient fibrin formation resulting in prolonged bleeding.

Several decades ago the mean age of death of haemophiliacs was less than 20 years; between 1950 and 1970, however, research into the factor VIII disorder led to the treatment of haemophilia A, first with whole plasma and, later, with plasma concentrates. Human plasma is both an inefficient and expensive source of this labile protein which is present at only $100\text{--}200\text{ ng ml}^{-1}$ (ref. 3). Pharmaceutical products currently available are highly impure ($<1\%$ factor VIII) and some are produced

Table 1 Assays of recombinant factor VIII activity

Sample	Coatest assay (units ml^{-1})	Radioimmune assay (units ml^{-1})	
		C7F7 Ab	C10 Ab
62.2 supernatant	0.07	0.28	0.34
62.2 supernatant + C7F7	<0.01	—	—
62.2 supernatant + Synbiotic	<0.01	—	—
Control supernatant	<0.01	<0.001	<0.001
62.2 cell lysate	—	0.1	0.1

Culture supernatant from recombinant factor VIII producing 62.2 cells, with and without inhibiting monoclonal antibodies, was subjected to the chromogenic 'Coatest' or to radioimmune assays. The Coatest assay (Helena Labs) was performed by diluting $5\text{ }\mu\text{l}$ of 62.2 supernatant with $45\text{ }\mu\text{l}$ 0.05 M Tris, pH 7.3 containing 0.2% bovine serum albumin (BSA) and incubating this mixture with or without either $5\text{ }\mu\text{g}$ C7F7 or Synbiotic factor VIII monoclonal antibodies. Both these antibodies react with the M_r 80,000 subunit of plasma-derived factor VIII. After 5 min at 24°C , $50\text{ }\mu\text{l}$ of a mixture containing factor IXa, factor X and phospholipid was added and samples incubated for 5 minutes at 37°C . $50\text{ }\mu\text{l}$ of each of 25 mM CaCl_2 and S-2222 chromogenic substrate was added and after 10 min at 37°C the reaction stopped by the addition of 50% acetic acid. The assay was repeated with control cultures and calibrated with normal human plasma. One factor VIII unit is equivalent to the amount of factor VIII in 1 ml normal human plasma. Radioimmune assays: The 96 wells of a microtitre dish were coated overnight with $100\text{ }\mu\text{l}$ 50 mM NaHCO_3 buffer, pH 9.6, containing 2.5 mg l^{-1} of C.C. antibody purified by protein A-Sepharose chromatography. The wells were washed three times with $200\text{ }\mu\text{l}$ of phosphate-buffered saline (PBS) containing 0.05% Tween 20, blocked with $200\text{ }\mu\text{l}$ of PBS containing 0.1% gelatin and 0.01% merthiolate for 1–2 h, rewashed as before and $100\text{ }\mu\text{l}$ of sample added and incubated overnight. The wells were washed and $100\text{ }\mu\text{l}$ of ^{125}I labelled C10 or C7F7 antibody ($1,000\text{ c.p.m. }\mu\text{l}^{-1}$) added and incubated for 6–8 h. The wells were washed again and counted. Standard curves were derived from samples of normal plasma. Antibodies used in assays and columns were: C.C., a polyclonal antibody derived from the plasma of a severely affected haemophiliac⁴⁰; C8 and C10, neutralizing monoclonals binding to the M_r 210,000 and 90,000 fragments of factor VIII, supplied by A. H. Goodall, Department of Immunology, Royal Free Hospital, London⁴¹; C7F7, a neutralizing monoclonal binding to the M_r 80,000 fragment of factor VIII, derived at Genentech by Badora Streck and an 80,000 binding monoclonal obtained from Synbiotic Corporation.

from pooled plasma lots derived from thousands of donors. Thus, these products are associated with a variety of serious complications caused by protein precipitates as well as the possible contamination by adventitious agents such as hepatitis virus and the agent responsible for acquired immune deficiency syndrome (AIDS).

Factor VIII is a molecule of relative mass (M_r) 300,000 (ref. 4). In attempts to produce pure homogenous factor VIII, we have identified clones containing the factor VIII gene using oligonucleotide probes based on the protein sequence data reported in an accompanying paper⁵. We also show that cloned full-length factor VIII protein coding sequences assembled into a plasmid direct the synthesis of biologically active factor VIII when transfected into cultured mammalian cells.

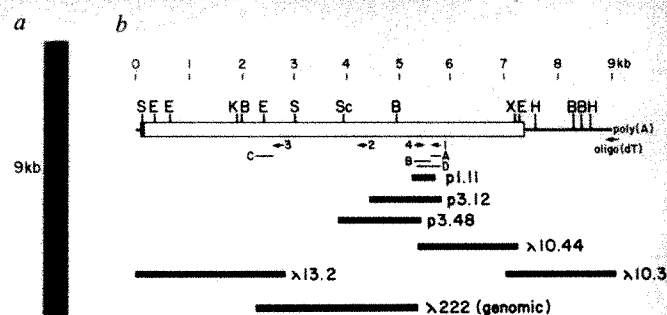
Isolation of genomic clones

Two factors contributed to our decision to initially screen recombinant genomic libraries (rather than RNA-derived cDNA libraries) for the factor VIII gene. First, the site of synthesis of factor VIII is uncertain, although the liver is considered the most likely source^{6–10}. Second, the concentration of factor VIII in plasma is extremely low (200 ng ml^{-1}), about $1/2,000,000$ the molar concentration of serum albumin. Thus, it was not clear that cDNA libraries made from mRNA of a given tissue would yield any factor VIII clones.

Therefore we adopted a strategy of first identifying a genomic clone corresponding to a sequenced portion of the factor VIII protein. Then several paths could be followed. An exon-containing restriction fragment could be used to identify a

Fig. 2 cDNA cloning procedure. **a**, 25 μ g poly(A)⁺ RNA from the AL-7 cell line was electrophoresed in a 1% agarose gel containing 6% formaldehyde⁴², transferred to nitrocellulose and hybridized in 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 10% dextran sulphate, 50% formamide, 0.1% SDS, 0.1% sodium pyrophosphate, 0.1 mg ml⁻¹ *E. coli* tRNA at 42°C overnight with ³²P-labelled probe prepared from the 189 bp *Stu*I/*Hinc*II genomic clone fragment containing exon 16 sequence. Wash conditions were 0.2 $\times$ SSC, 0.1% SDS at 42°C. This RNA was 0.0001–0.001% of the total cytoplasmic poly(A)⁺ RNA in the AL-7 cell line by comparison with control DNA dot-blot hybridizations⁴³. **b**, Factor VIII mRNA; open bar, mature protein coding region; hatched area, signal peptide coding region; adjacent lines, untranslated regions of the message. Left, the 5' end of the mRNA. Below the mRNA line are the six cDNA clones and the genomic clone containing exon 14 from which the full length factor VIII clone was assembled. cDNA synthesis primers 1–4 and oligo(dT) are shown with arrows depicting the direction of synthesis for which they are primed. Primer 1, 5' CAGGTCAA-CATCAGAG; primer 2, 5' TATCTGTGTGAGGGTG; primer 3, 5' AACTCTGTGTGTCAG; primer 4, 5' TATTGCTGCAGTGGAG. Probe A, 189 bp *Stu*I/*Hinc*II fragment from exon 16; probe B, 361 bp *Sau*3A1/*Stu*I fragment of p1.11; probe C, 324 bp *Hinc*II fragment from exon 14; probe D, 419 bp *Pst*I/*Hinc*II from p1.11. The original 36-mer probe 8.3 is located near the primer 1 region. Restriction endonuclease sites in the corresponding DNA are indicated by: B, *Bam*HI; E, *Eco*RI; H, *Hpa*I; K, *Kpn*I; S, *Sac*I; Sc, *Sal*I; X, *Xba*I.

Methods: the first cDNA clones were isolated by priming cDNA synthesis with primer 1 from exon 16. From 5 μ g AL-7 poly(A)⁺ RNA, approximately 100,000 *E. coli* transformants were obtained by introducing the double-stranded cDNA into pBR322 by the oligo(dC:dG) tailing method⁴². These clones were screened by hybridization to probe A⁴⁴ and a single clone containing a 447 bp cDNA insert (p1.11) (including the 5' 104 bp of exon 16) was isolated. The remaining sequences of p1.11 extended 5', including exon 15 and part of exon 14. Exon 14 had been found earlier by hybridization with an oligonucleotide probe based on protein sequence data¹⁶. Hybrid selection was used to further enrich for factor VIII specific sequences in AL-7 mRNA. To avoid degradation of the relatively large factor VIII transcripts, single-stranded cDNA was initially synthesized on a preparative scale and subjected to hybrid selection prior to conversion to double-stranded molecules. First-strand synthesis was performed on 200 μ g AL-7 poly(A)⁺ RNA with primer 1 and the resulting product was hybridized with an exon 16 DNA fragment (probe A), denatured and immobilized on DBM-cellulose paper⁴². After elution, 0.5–1.0 ng of single-stranded material was recovered and used to prepare 12,000 *E. coli* transformants. 29 clones were obtained by hybridization to probe B which did not overlap the DNA fragment used for hybrid selection. This represents a 240-fold enrichment of desired clones compared with the first cDNA library. DNA sequence shows that the two longest clones (p3.12, p3.48) extended about 1,500 bp further 5' than p1.11. Primer 3, located near the 5' end of exon 14, was used to isolate the N-terminal coding sequences for factor VIII. Double stranded cDNA was prepared and ligated to synthetic complementary *Eco*RI adaptors, 5'pCCTTGACCGTAAGACATG and 5'AATTCATGTCTTACGGTCAAGG. Upon annealing, these adaptors have a phosphorylated blunt end which will be covalently joined to the cDNA, as well as a cohesive *Eco*RI terminus with a 5'-hydroxyl group which cannot self-ligate, thus avoiding the need for methylation of the cDNA and subsequent *Eco*RI digestion. Approximately 1.5 $\times$ 10⁶ recombinant phage were obtained from 1 μ g AL-7 poly(A)⁺ RNA with primer 3 following removal of excess adaptor, insertion into λ gt10 and *in vitro* packaging^{42,45}. Forty-six clones were identified by hybridization with probe C. Several of the clones contained cDNA inserts which extended about 2,700 bp 5' of primer 3 and the DNA sequence of the two longest clones (λ 13.2 and λ 13.27) revealed the 5' terminus of the protein coding region (Fig. 3). (Primer 2 was only used subsequent to the cloning and expression described here to obtain cDNA sequence data corresponding to the region of genomic clone λ 222 containing exon 14.) To isolate the coding region for the C-terminus of factor VIII, a cDNA library was prepared from oligo(dT) primed AL-7 RNA. A 16-mer corresponding to the mRNA sequence found 400 bp upstream of the 3' end of exon 16 (primer 4) was included in the second strand reaction to increase its specificity. Four of a total 3 $\times$ 10⁶ λ gt10 clones hybridized with a fragment from p3.12 (probe D) located 3' of primer 4. DNA sequence analysis of the longest clone, λ 10.44, indicated that the cDNA insert began at primer 4 and extended for nearly 1,800 bp 3', but had not reached the end of the coding region. To identify clones containing the 3' end of the mRNA, the oligo(dT)-primed library was rehybridized with a probe prepared from the insert of λ 10.44. Twenty-four additional clones were recovered and the two longest of these (λ 10.3 and λ 10.9) were sequenced. Both clones overlapped λ 10.44 as expected and extended an additional 1,900 bp to the poly(A) tail.



suitable tissue or cell source of factor VIII by RNA blot hybridization. The mRNA from this source would be used to generate cDNA clones which could be screened by cloned genomic fragments. Concurrently, overlapping genomic clones could be isolated encompassing the entire factor VIII gene. Even if a direct mRNA source were not identified, exons from the genomic clones could be found and spliced together by simian virus 40 (SV40) recombinant 'exon expression' plasmids. In fact, we used information derived from all three paths.

A genomic library enriched for the X chromosome was constructed from DNA isolated from a lymphoblast cell line derived from an individual with four X chromosomes (karyotyped 49,XXXXY). To identify factor VIII clones, a series of oligonucleotide probes based on portions of the human factor VIII protein sequence⁵ was prepared by chemical synthesis. The oligonucleotide probes were of two types: unique long probes of 30–100 nucleotides based on codon usage analysis and mixtures of short probes of 14–20 bases specifying all possible combinations for each codon choice^{11–13}. The utility of probe 8.3 (Fig. 1 legend) was first tested by genomic blot hybridizations with normal male (1X) and 49,XXXXY (4X) DNA. Even at the highest stringency (1 $\times$ SSC, 46°C), a single *Eco*RI or *Bam*HI band was observed which had an intensity ratio of about 1:4 in the 1X and 4X lanes, as expected for the X-linked factor VIII gene (Fig. 1).

Next, 500,000 phage of the 4X genomic library were screened with probe 8.3 and 15 clones representing eight distinct overlapping recombinants were isolated and characterized (Fig. 1). The DNA sequence of the region hybridizing with the 8.3 probe included two continuous matches of 14 and 10 base pairs (bp) and had an overall homology of 83%. The first 10 of the 12 amino acid residues of the peptide fragment agreed with those deduced from the DNA sequence of the recombinant clones. The DNA sequence also predicted a lysine residue preceding

the peptide, as would be expected for the product of a tryptic digest. The final two residues did not match the DNA-predicted sequence; the DNA at this junction, however, contained a probable RNA splice donor sequence^{14,15} followed shortly by stop codons in all three possible reading frames.

Inspection of the open reading frame immediately 5' of the probe homology revealed matches with the protein sequence of several additional tryptic peptides from the *M_r* 80,000 factor VIII fragment⁷. The identity between highly purified factor VIII

Fig. 3 Sequence of human factor VIII gene, with nucleotides numbered at the left of each line. One, the A of the translation initiation codon ATG. Negative numbers, 5' untranslated sequence. (mRNA mapping experiments suggest that factor VIII mRNA extends ~60 nucleotides further 5' than position -109 shown here¹⁶.) The predicted protein sequence is shown above the DNA. Numbers above the amino acids are S1–19 for the predicted signal peptide, and 1–2,332 for the predicted mature protein. 'Op', the opal translation stop codon TGA. The 3' polyadenylation signal AATAAA¹⁸ is underlined and eight residues of the poly(A) tail (found in clone λ c10.3) are shown. The sequence homologous to the synthetic oligonucleotide probe 8.3 is also underlined (nucleotides 5,557–5,592). Selected restriction endonuclease cleavage sites are shown above the appropriate sequence. The entire factor VIII sequence was verified after assembly of cDNA and genomic clones in an expression plasmid. The complete DNA sequence of the protein coding region of the human factor VIII gene was also determined from the genomic clones we have described¹⁶. cDNA clones have been isolated covering all of the sequence shown except for nucleotides 3,042–3,216. The cDNA and genomic clones differ at only two positions: nucleotide 3,780 (underlined) is C in the genomic clone and G in cDNA (changing amino acid 1,241 from Glu to Asp), and nucleotide 8,728 (underlined) in the 3' untranslated region is A in the genomic clone and G in cDNA. DNA sequence analysis was performed by the dideoxy chain termination method using restriction fragments subcloned into single-stranded M13 vectors^{46,47}.

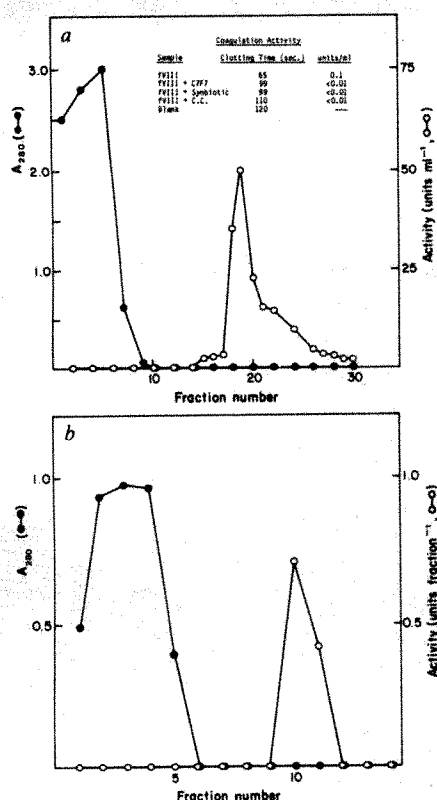


Fig. 5 Immunoaffinity chromatography of recombinant factor VIII. *a*, Chromatography on C7F7-Sepharose; *b*, chromatography on von Willebrand factor-Sepharose.

Methods: Serum free culture media of transformed cell line 62.2 containing 400 units of factor VIII was first adsorbed onto DEAE-sepharose (Pharmacia). The gel was washed subsequently with 0.05 M imidazole pH 6.9 containing 0.15 M NaCl, 0.01 M CaCl₂ and 0.02 M glycine ethyl ester; factor VIII was eluted with the same buffer containing 0.1 M CaCl₂. Material obtained from DEAE-sepharose was then passed through a factor VIII monoclonal antibody column. The column was prepared by covalently linking 2 mg factor VIII monoclonal antibody C7F7 to 1 ml of Affi-gel 10 (Bio-Rad). After loading, the column was washed sequentially with 20 volumes of 0.02 M imidazole pH 6.4 containing 0.15 M NaCl and 0.01 M CaCl₂ and 10 volumes of the same buffer containing 0.5 M NaCl. Factor VIII activity was eluted with 0.02 M imidazole pH 6.4 containing 1 M KI and 20% ethylene glycol and measured by the Coatest assay (Table 1). This figure depicts chromatography performed with the monoclonal antibody C7F7, known to react with the M_r 80,000 plasma-derived factor VIII protein fragment. Comparable results were obtained with a column using the C8 monoclonal antibody which binds to the M_r 210,000 and 90,000 fragments of factor VIII. The table inset of *a* shows the neutralization of coagulant activity by factor VIII antibodies. Partially purified recombinant factor VIII was dialysed into 0.05 M Tris pH 7.4 containing 0.15 M NaCl, then diluted with the same buffer containing 0.2% BSA. 50 µl aliquots of this sample were preincubated alone, with human factor VIII monoclonal antibodies (5 µg of either C7F7 or a human factor VIII monoclonal purchased from Synbiotics, Inc.), or with 5 µl of serum derived from a patient with a high factor VIII antibody titre⁴⁰ (C.C.) for 5 min at 24 °C. Factor VIII activity was then determined by coagulation assay. Briefly, 50 µl platelin (General Diagnostics) was incubated with 50 µl factor VIII deficient plasma for 8 min at 37 °C. 50 µl sample was then added and after 30 s at 37 °C, 50 µl 25 mM CaCl₂ was added and the clotting time measured. Supernatant from 62.2 cell cultures containing ~2 units of factor VIII activity was passed through a von Willebrand factor-sepharose column at 30 ml h⁻¹. The von Willebrand factor used here was purified as described previously⁵¹. von Willebrand factor-sepharose was prepared by covalently linking the protein to Affi-gel 10 (1 mg ml⁻¹ gel). After loading the column, it was washed with 5 volumes 0.05 M imidazole pH 6.9 containing 0.15 M NaCl, 0.01 M CaCl₂ and 0.02 M glycine ethyl ester. Factor VIII activity was eluted with the same buffer containing 0.25 M CaCl₂ and fractions assayed with the Coatest assay.

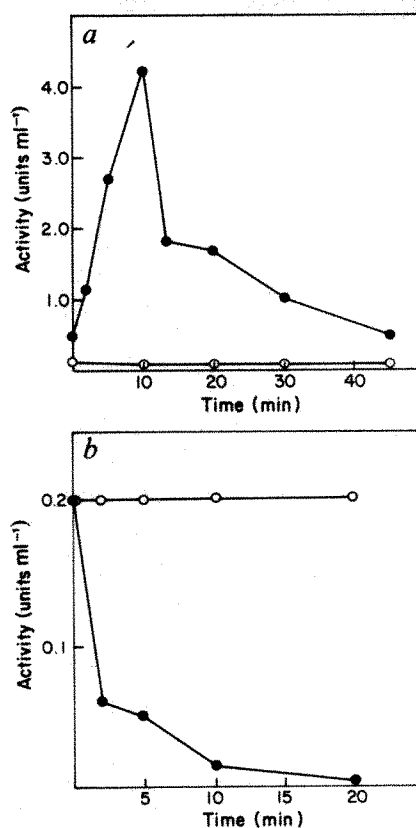


Fig. 6 Effect of thrombin and protein C on factor VIII activity.

a, Recombinant factor VIII obtained from immunoaffinity chromatography was dialysed into 0.05 M Tris pH 7.4 buffer containing 0.15 M NaCl and diluted to a concentration of 0.1 units ml⁻¹ with the same buffer containing 0.2% BSA (TBS buffer). Aliquots of this sample were then incubated either with thrombin at 0.01 units ml⁻¹ (closed circles) or without thrombin (open circles) for the times shown at 37 °C. At the given time points, factor VIII activity was determined by the coagulation assay described in Fig. 5 legend. Thrombin at the concentration used here did not decrease background clotting times of samples in which factor VIII was omitted. *b*, Recombinant factor VIII was diluted into TBS buffer to a concentration of 0.2 units ml⁻¹. 5 µl rabbit brain cephalin (Sigma) and CaCl₂ to 5 mM were added to 50 µl aliquots of this sample. Subsequently these samples were incubated with activated protein C (provided by Walter Kiesel, University of Washington) at 0.4 mg ml⁻¹ (closed circles) or with no addition (open circles) for the times shown at 37 °C. Residual activity was then determined as described above.

dihydrofolate reductase cDNA transcribed by an SV40 early promoter to provide a selectable marker when expressed in mammalian cells²⁵. The plasmid was introduced into a hamster kidney cell line by the calcium-phosphate coprecipitation method^{25,26}; we chose one clone, 62.2, for further study.

To characterize the expression of factor VIII in the 62.2 cell line, RNA was extracted from cells and analysed by Northern blot hybridization. A predominant 9 kb RNA from 62.2 cells, but not from untransfected control cells, was found to hybridize both with a probe specific for factor VIII and with one containing hepatitis B surface antigen 3' sequences (data not shown). The intensity of the hybridizing signal with 62.2 RNA was 200 times greater than that observed with RNA prepared from the AL-7 cell line. Radioimmune assays for factor VIII were also performed on the 62.2 supernatant and cell lysate (Table 1). Monoclonal antibodies directed against the N-terminal M_r 210,000 fragment (C10) or the C-terminal M_r 80,000 fragment (C7F7) of serum-derived factor VIII detected a 300-fold increase in factor VIII cross-reacting activity in 62.2 cells over that found in control cells (Table 1).

Biochemical properties

The activation of natural factor X by factor IXa is dependent on factor VIII, which is the basis of the 'Coatest' assay we have used to estimate factor VIII in cell supernatants; we find significant activity in 62.2 supernatants but not in those from control cells not transfected (Table 1). Supernatant from 62.2 cells alone did not catalyse the cleavage of the chromogenic substrate. In addition, incubation of 62.2 cell supernatants with either of two monoclonal antibodies directed against the M_r 80,000 fragment of factor VIII (C7F7 and Synbiotic) completely neutralized the observed activity (Table 1).

To characterize further the factor VIII secreted by 62.2 cells, partially purified protein was tested for the ability to reduce the clotting time of plasma from patients with haemophilia A. Protein from 62.2 cell supernatants was affinity purified on a monoclonal antibody column under the same conditions used factor VIII isolated from human plasma (Fig. 5a). Diluted protein samples from the immunoaffinity column reduced the clotting time of plasma from a haemophilia A patient from 120 s to 65 s (equivalent to the clotting time for a 1:10 dilution of normal plasma). This activity was neutralized by the C7F7 and Synbiotic monoclonal antibodies directed against the M_r 80,000 fragment of factor VIII as well as by the patient-derived polyclonal antibody, C.C. (Fig. 5a).

Factor VIII circulates in plasma as a complex with the von Willebrand protein^{27,28}. Factor VIII/von Willebrand protein complexes are dissociated by high ionic strength or by calcium chloride containing buffers²⁹⁻³²; factor VIII derived from plasma can be bound to von Willebrand factor-Sepharose and eluted with CaCl_2 ³³. Recombinant factor VIII bound quantitatively to such an affinity column and could be completely eluted from the column with CaCl_2 , like plasma-derived factor VIII (Fig. 5b).

The coagulant activity of plasma-derived factor VIII is increased markedly by incubation with small amounts of the serine protease thrombin³⁴⁻³⁸. Thrombin activation probably results from the cleavage of specific peptide bonds in factor VIII⁵. After activation, continued incubation with thrombin leads to further degradation of the protein with a concomitant loss of coagulant activity. Recombinant factor VIII is affected by thrombin in a similar manner. The coagulation activity of the affinity purified protein from 62.2 cells was stimulated initially more than 40-fold by thrombin treatment (Fig. 6a). After longer incubation with thrombin, the activity substantially decreased (Fig. 6a). Plasma-derived factor VIII has also been shown to be inactivated by activated protein C^{34,39}, a vitamin K-dependent serine protease found in plasma, possibly functioning as a physiological regulator of coagulation. Incubation of the affinity purified 62.2 factor VIII with activated protein C resulted in the rapid loss of coagulation activity, as expected (Fig. 6b).

Conclusions

We have demonstrated by several criteria that the DNA sequences isolated here encode human factor VIII. Thus, genomic blots of male and 4X DNA hybridize with the 1:4 ratio expected for an X-linked gene. Moreover, the amino acid sequence derived from factor VIII preparations and the primary structure of the protein predicted by the cloned sequence are essentially identical⁵. Indeed, purified factor VIII preparations from plasma usually consist of a set of fragments between M_r 90,000 and 210,000 with very similar tryptic peptide maps and another, with M_r of 80,000, with a distinct map. Comparison of tryptic peptide sequences obtained from both classes of plasma-derived polypeptides⁵ with the DNA sequence of the cloned gene demonstrates that the M_r 210,000 and 80,000 polypeptides represent the N-terminal and C-terminal portions, respectively, of a single 2,332 amino acid mature polypeptide gene product. This protein presumably corresponds to the non-reducible M_r 330,000 species observed in purified factor VIII preparations⁴. The discrepancy between the molecular mass of single-chain plasma-derived factor VIII and that calculated for the cloned protein may be accounted for by the 25 potential

asparagine-linked glycosylation sites predicted by the latter. The peak of factor VIII activity found upon thrombin incubation correlates with the enhanced presence of the M_r 90,000 and a 70,000 fragment²¹. We have demonstrated that the 90,000 fragment corresponds to the amino terminal portion of the full-length molecule, that it derives from carboxy-terminal cleavage of the 210,000 fragment and that it contains the A1 and A2 repeated domains. The 70,000 species derives from the carboxy-terminus of the molecule following cleavage of the 80,000 fragment, including the A3, C1 and C2 domains⁵. Thus, removal of the unique B domain which connects the 90,000 and 80,000 fragments is associated with activation of the protein.

The recombinant factor VIII from the supernatants of cells transfected with the reconstructed gene corrects the coagulation defect of factor VIII-deficient plasma. A more stringent test of the recombinant protein was provided by the 'Coatest' assay, which measures the ability of factor VIII to accelerate the rate at which factor IXa proteolytically activates factor X in part of the intrinsic pathway of blood coagulation. The ability of several antibodies directed against factor VIII to neutralize these activities rules out the possibility that the cloned protein corresponds to other factors, such as the tissue factor/factor VII complex of the extrinsic coagulation pathway, also known to activate factor X.

It is anticipated that the availability of recombinant factor VIII from serum-free cell supernatants will further aid the study of structural modifications made on factor VIII by other components of the coagulation cascade. Such studies will be facilitated by the opportunity to create alterations in the structure, processing and activity of factor VIII by *in vitro* mutagenesis and heterologous expression of the cloned DNA sequences. Moreover, it is hoped that recombinant factor VIII will provide a safer and more plentiful therapy for individuals afflicted with haemophilia A.

We thank D. Hansen, B. Baer, U. Edman, T. Goralka, A. Kleiss, D. Leung, B. O'Connor and J. Souder for contributing to the work described here. Identification of cell line producing factor VIII RNA, cloning and assembly of factor VIII cDNA was performed by D. Capon. We also thank Genentech's Assay Development Group, Monoclonal Antibody Laboratory and the Organic Chemistry Laboratory. We appreciate the helpful discussions and continued support of D. Goeddel, A. Levinson, D. Martin, A. Ullrich, G. Hooper and R. Swanson and thank J. Arch and A. Gray for preparing the manuscript and figures. This work was supported by Genentech, Inc. and Speywood Laboratories Ltd.

Received 9 August; accepted 27 September 1984.

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Structure of human factor VIII

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The deduced amino acid sequence of human factor VIII, obtained from the DNA sequence, predicts a mature polypeptide of 2,332 amino acids containing a triplicated domain structure. The polypeptide has 35% sequence homology with the copper-binding plasma protein, ceruloplasmin. Determination of the thrombin cleavage sites in plasma-derived factor VIII polypeptides allows prediction of the domains involved in the associated activation and inactivation of the protein.

PREPARATIONS of factor VIII/von Willebrand factor complex¹⁻¹² contain four closely related properties or activities: factor VIII coagulant activity, an antigen associated with the factor VIII coagulant activity, platelet adhesion promoting activity and an antigen precipitated by antisera raised against the purified complex (factor VIII-related protein). Factor VIII separated from the complex has associated trace amounts of protein, is unstable and consists of multiple polypeptide chains¹³⁻¹⁷, hindering detailed characterization studies.

The purification of human factor VIII by affinity to a monoclonal antibody directed against the coagulant activity of factor VIII¹⁸, has allowed characterization of the protein fragments of factor VIII or thrombin-activated factor VIII by partial amino acid sequence analysis. This sequence information has been used to isolate cDNA and genomic clones encoding human factor VIII^{19,20}. The protein sequence deduced from these clones, together with an analysis of the cleavage products associated with the activation of factor VIII by thrombin, allows the assignment of thrombin cleavage sites and the identification of most of the polypeptide fragments present in highly purified factor VIII preparations. The factor VIII sequence exhibits striking homology with the plasma copper-binding protein ceruloplasmin, suggesting novel biochemical activities for factor VIII as well as a role for metal ions other than calcium in the blood coagulation cascade.

Analysis of plasma factor VIII

Preparations of human factor VIII¹⁸ purified over 300,000-fold from plasma contained several proteins of relative molecular mass (M_r) 210,000-80,000 (Fig. 1A). These protein bands were not connected by disulphide links because samples analysed under non-reducing conditions gave a similar pattern (data not shown). To determine the relationship of these multiple polypeptide chains, we analysed them by tryptic peptide mapping. The preparation used in Fig. 1B contained a protein of M_r 240,000, producing a peptide map which did not show identity with the other proteins of the mixture (Fig. 1B, a), and has been found to be a von Willebrand factor subunit (data not shown). Peptides of M_r 90,000-210,000 all had a common tryptic map, indicating that they are derived from the same or closely related polypeptide chains (Fig. 1B, b-f). Furthermore, Western blot analysis of the factor VIII preparations demonstrated that a factor VIII-specific monoclonal antibody¹⁸ reacted with the M_r 90,000-

210,000 polypeptides (data not shown). Two very similar patterns generated by the proteins of M_r 80,000 and 70,000 had a different peptide map (Fig. 1B, g, h). These results demonstrate that the fragments of M_r 90,000-210,000 are structurally related and could be pooled and treated as one polypeptide chain. The protein represented by the band of M_r 80,000 (and 70,000 when present) was analysed separately.

The purified factor VIII preparations were fractionated by gel filtration on a TSK 4000SW HPLC column; analysis of the resulting fractions demonstrated the effective separation of M_r 240,000 protein, the polypeptides of M_r 90,000-210,000 and the M_r 80,000 fragment (Fig. 2B). We performed amino acid sequence analysis on peptides generated from the M_r 80,000 protein, the polypeptide pool of M_r 90,000-210,000 and a fragment of M_r 90,000 from limited thrombin digestion of the M_r 90,000-210,000 pool. After digestion of each sample with trypsin, the resulting peptides were separated by reverse-phase HPLC and sequenced. The peptide sequence AWAYFSDVDLEK, used to prepare synthetic DNA probes identifying factor VIII genomic DNA clones, is indicated in Fig. 2C.

Structure of factor VIII protein

The molecular cloning of the entire factor VIII coding region is described in an accompanying paper¹⁹. The 2,351-amino acid sequence for factor VIII, deduced from the nucleotide sequence of these clones, is shown in Fig. 3. The first 19 amino acids of the sequence comprise the signal sequence for factor VIII, based on peptide sequence analysis of a fragment derived from the M_r 90,000-210,000 polypeptide pool. The N-terminal sequence of this M_r 30,000 fragment, obtained as a thrombin digest product of the M_r 90,000-210,000 pool, is identical to the first 12 amino acids which follow the predicted factor VIII leader sequence (see Fig. 5). This presequence exhibits a core of 10 hydrophobic amino acids flanked by two charged residues, a structure which conforms to that observed for the leader sequences found in most secreted proteins²¹. The mature protein contains 2,332 amino acids (calculated M_r 264,763).

The availability of the complete factor VIII sequence reveals the organization and identity of the tryptic peptides obtained from the pools of separated plasma-derived factor VIII fragments. Essentially all tryptic peptide sequences determined from the M_r 90,000-210,000 protein pools are located in the amino-

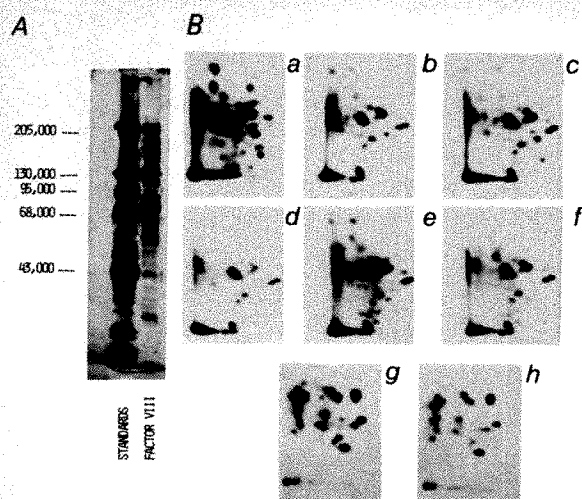


Fig. 1 A, Purified human factor VIII analysed by SDS-polyacrylamide gel electrophoresis. B, Two-dimensional tryptic mapping of factor VIII polypeptide chains. The resulting tryptic patterns of proteins of M_r : a, 240,000; b, 210,000; c, 170,000; d, 150,000; e, 120,000; f, 100,000; g, 80,000; and h, 70,000 are shown.

Methods: The purified protein¹⁸ was reduced and analysed in a 5–10% polyacrylamide gradient gel in the presence of SDS by the procedure of Laemmli³⁷. The molecular weights of the protein standards are shown (myosin, β -galactosidase, phosphorylase B, bovine serum albumin and ovalbumin). The bands were detected using the silver stain procedure of Morrissey³⁸. 1 μ g of a factor VIII preparation was denatured in 1% SDS and labelled with 300 μ Ci ^{125} I for 15 min using the iodobead procedure³⁸. Labelled polypeptides were located on dried SDS-polyacrylamide gels by autoradiography and digested by incubation of gel slices with 10 μ g trypsin in 0.1 M ammonium bicarbonate buffer for 6 h at 37°C. After repeated lyophilizations, peptides were dissolved in 8.8% formic acid and a portion was subjected to thin-layer electrophoresis in the same buffer (400 V for 45 min) on pre-coated TLC-cellulose platters (E. Merck, Darmstadt, FRG). For the second dimension, peptides were separated by ascending chromatography in *n*-butanol/pyridine/glacial acetic acid/water, 75:50:15:60 (v/v). The plates were then subjected to autoradiography.

terminal half of the molecule, whereas those sequences obtained for the M_r 80,000 fragment are found at the carboxy-terminus of the factor VIII sequence (unpublished results). The most carboxy-terminal tryptic peptides identified for the M_r 90,000–210,000 pool gave the sequences GEFT and –QEE, beginning at positions 1,155 and 1,194, respectively. This shows that the M_r 210,000 fragment consists of a protein of $M_r \geq 135,000$ containing 14 potential asparagine-linked glycosylation sites. The location of the M_r 80,000 fragment of factor VIII is delineated by two peptide sequences which define a stretch of ~680 amino acids. The first of these was obtained from the amino-terminal sequence of the M_r 80,000 fragment beginning at position 1,649 (see Fig. 5), whereas the second corresponds to a tryptic peptide, MEVLGCEAQDL, 12 amino acids from the C-terminus predicted by the DNA sequence. Thus, there is no significant removal of C-terminal sequences from the plasma-derived molecule. The failure to recover tryptic peptide sequences from the region between position 1,200 and the M_r 80,000 fragment is probably due to the relatively low concentration of the M_r 210,000 species in the M_r 90,000–210,000 fragment pool. This position should therefore be considered the minimal C-terminal extent of the M_r 210,000 protein.

Computer-aided analysis of the factor VIII protein sequence revealed two types of internal homology: the first consists of a triplicated segment (A domain) found at positions 1–329, 380–711 and 1,649–2,019 of the mature polypeptide (Fig. 4); the second and third domains of the triplication are separated by a region of 983 amino acids (B domain) extremely rich in potential asparagine-linked glycosylation sites. In addition, an unrelated duplication of 150 amino acids is found at the C-terminus of the molecule (C domain). The A domains have ~30% amino acid homology, whereas the C domains are ~40% homologous. Most

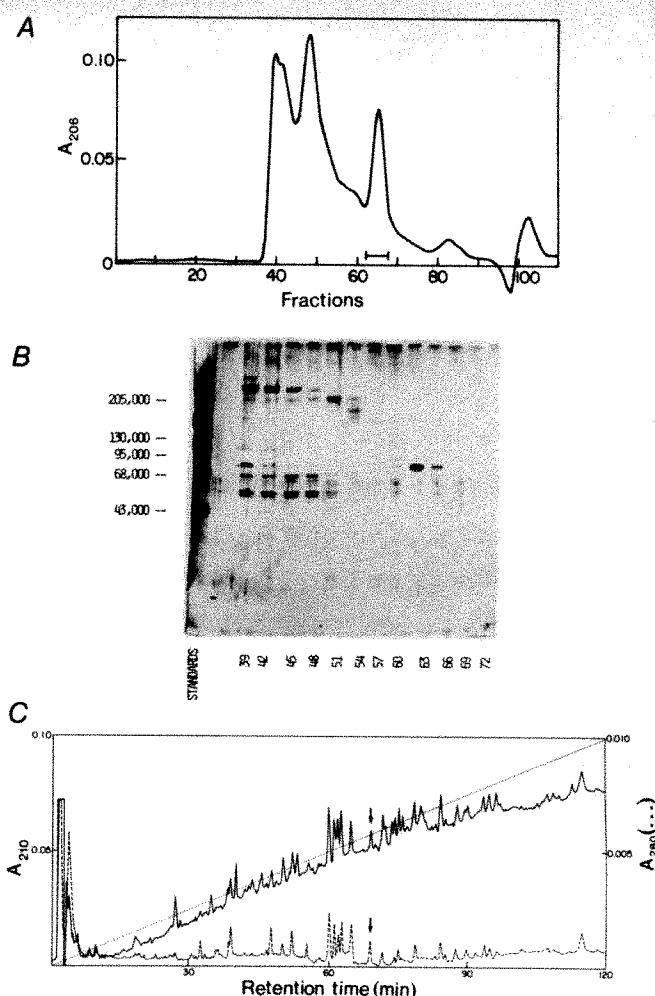


Fig. 2 Purification of factor VIII polypeptides. A, Fractionation of proteins by TSK 4000 HPLC. B, SDS-polyacrylamide gel analysis of fractions from TSK 4000 chromatography. C, Reverse-phase HPLC separation of tryptic peptides of M_r 80,000 protein.

Methods: A, Human factor VIII preparations¹⁸ were dialysed into 1% ammonium bicarbonate containing 0.1% SDS. The samples were lyophilized and stored at –20°C until use. The samples were reconstituted in distilled water and applied to a TSK 4000 SW column (0.75 $\times$ 50 cm; Alltech Associates, Deerfield, Illinois) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS. Samples of ~0.15–0.25 ml were injected and the column developed isocratically at a flow rate of 0.5 ml min^{–1}. Absorbance was monitored at either 206 nm or 280 nm and fractions (0.2 ml) collected. Fractions across the profile were reduced and analysed by SDS-polyacrylamide gel electrophoresis as described in Fig. 1 legend. The protein of M_r 80,000 was pooled for tryptic digestion as shown in A. C, The TSK 4000 SW purified protein of M_r 80,000 (0.8 nM) was dialysed under a nitrogen atmosphere overnight against 0.36 M Tris-HCl, pH 8.6, containing 8 M urea, 3.3 mM EDTA and 10 mM dithiothreitol, DTT (final vol. 1.5 ml). The protein was alkylated by adding 15 μ l of 5 M iodoacetic acid (dissolved in 1 M NaOH). The reaction was allowed to proceed for 35 min at room temperature in the dark and was quenched by adding DTT to a final concentration of 100 mM. The protein solution was dialysed against 8 M urea in 0.1 M ammonium bicarbonate for 4 h. The urea dialysis solution was changed over a period of 24 h to gradually reduce the urea concentration to a final level of 0.5 M. Trypsin was added at a weight ratio of 1:50 at 37°C for 12 h. HPLC separation of the resulting tryptic peptides was performed on a high-resolution Synchropak RP-P C-18 column (0.46 $\times$ 25 cm; 10 μ). The column was developed with a gradient of acetonitrile (1–70% in 200 min) in 0.1% trifluoroacetic acid. Absorbance was monitored at 210 nm and 280 nm. Each peak was collected and stored at 4°C until subjected to sequence analysis in a Beckman spinning cup sequencer with on-line phenylthiohydantoin amino acid identification⁴⁰. The arrow identifies the peptide (AWAYFSDVDLEK) resulting in identification of a factor VIII genomic clone^{19,20}.

Ceruloplasmin contains six copper atoms in three distinct types of coordination: two of type 1, one of type 2 and three electron paramagnetic resonance-nondetectable type 3 copper ions²⁶. The type 1 copper ions are thought to bind to the carboxy-terminal portion of the domains of ceruloplasmin (domain residues 240–350; Fig. 4) based on sequence homology with the type 1 copper-binding protein plastocyanin²⁷. The four amino acid side chains proposed as the ligands for the type 1

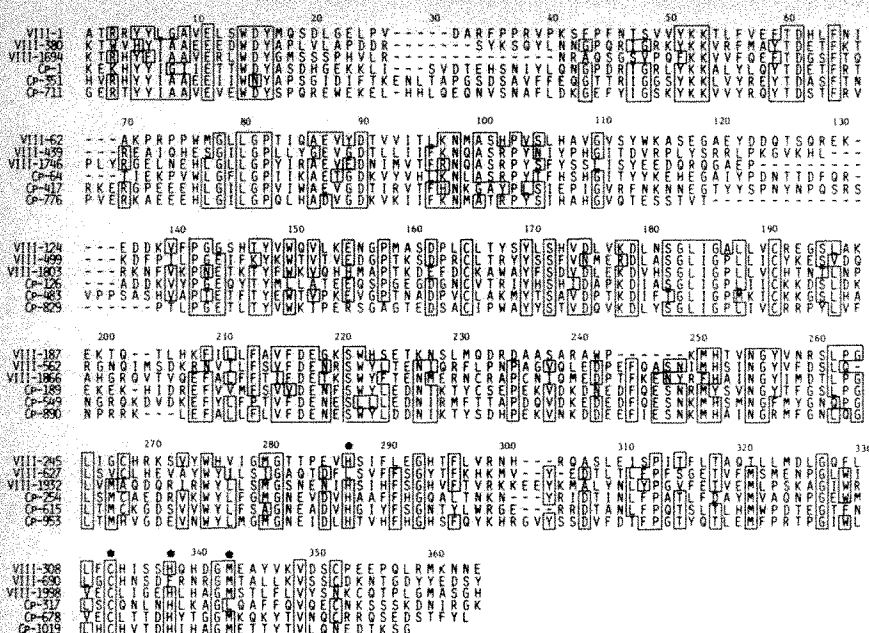


Fig. 4 Domain homology of human factor VIII and human ceruloplasmin²². Identical residues in four of the six domains are boxed. The protein is indicated to the left of the lines (VIII, factor VIII; Cp, ceruloplasmin) with the associated number indicating the position in the sequence of the first amino acid of the line. A domain residue numbering system is shown above the sequences. * Residues believed to be the ligands for type 1 copper (based on homology to plastocyanin²⁷).

copper atoms of ceruloplasmin are found in the first and third A domains of factor VIII (histidines 287 and 338, cysteine 333 and methionine 343; see Fig. 4). The conservation of the copper-ligand residues found in ceruloplasmin strongly suggests similar metal binding characteristics for factor VIII.

Thrombin cleavage

The coagulant activity of factor VIII is increased markedly by treatment with catalytic amounts of the serine protease thrombin²⁸. Thrombin activation of factor VIII is associated with a series of polypeptide cleavages¹³⁻¹⁷. Further incubation with thrombin leads to degradation of the protein with a concomitant loss of coagulant activity¹³⁻¹⁷. To understand the structural basis for these observations, the separated pools of factor VIII fragments were subjected to thrombin digestion and the resulting products characterized by SDS-polyacrylamide gel electrophoresis. Thrombin digestion of the *M*_r 80,000 protein resulted in a product of *M*_r 73,000 (Fig. 5A). Treatment of the polypeptide pool of *M*_r 90,000-210,000 led initially to the appearance of two bands of *M*_r 43,000 and 50,000 (Fig. 5A). Longer incubation with thrombin resulted in the conversion of the *M*_r 50,000 fragment to polypeptides of *M*_r 30,000 and 20,000 (data not shown). The *M*_r values of these thrombin digest fragments are similar to those generated by thrombin treatment of native factor VIII preparations^{13,15,16}. Amino-terminal sequence analysis was performed on the separated protein chains before and after thrombin digestion. The resulting sequences are compared with the corresponding amino acid sequences deduced from the factor VIII cDNA sequence¹⁹ in Fig. 5B; also shown are the potential cleavage sites found at the amino-terminus of the *M*_r 80,000 protein and that which separates the *M*_r 90,000 fragment from the carboxy-terminal portion of the *M*_r 210,000 protein. The sequence surrounding the latter potential cleavage site (position 740) is similar to the amino-terminal sequence of the *M*_r 70,000 protein (-PRSF---RH-) (Fig. 5B). There is no other consensus sequence that would predict the specificity of thrombin cleavage. A homologous stretch preceding the thrombin cleavage sites for the *M*_r 43,000 and 73,000 proteins is observed (Fig. 5B), but whether this homology determines thrombin specificity or simply reflects the internal duplication is uncertain. The most consistent sequence found at thrombin cleavage sites within factor VIII is an arginine residue followed by either serine or alanine. Other such sequences (-RS- or -RA-) do occur within the protein but are not cleaved, suggesting the possible involvement of secondary structure in thrombin specificity. The cleavage that frees the *M*_r 80,000 protein occurs at an arginine-glutamic acid sequence, probably not a thrombin-generated cleavage site. This cleavage occurs

quickly and is complete within the time required for isolation of the protein. The precursor factor VIII protein therefore may be cleaved to free the *M*_r 80,000 polypeptide by a protease other than thrombin.

Discussion

The structure of factor VIII revealed by the amino acid sequence predicted from the cloned cDNA and the structural characterization of polypeptide fragments described here are summarized in Fig. 6. The size of the factor VIII precursor moiety is consistent with the reported isolation of single-chain *M*_r 330,000 protein from plasma¹⁸ and supports the notion that the protein circulates as a high-molecular weight form that is readily cleaved in plasma and/or during isolation to a series of degradation products.

The primary structure of factor VIII exhibits three distinct types of structural domain, including a triplicated region of ~330 amino acids (A domains), a unique region of 980 amino acids (the B domain) and a carboxy-terminal duplicated region of 150 amino acids (C domains), which are arranged in the order A1-A2-B-A3-C1-C2 (Fig. 6). The A domains of factor VIII show significant homology to ceruloplasmin, consisting also of a triplicated structure of three A domains but lacking both B and C domains²²⁻²⁴. Particularly striking is the clustering of cysteine residues at similar locations within related structural domains of factor VIII (Fig. 6). The determination of disulphide pairings for ceruloplasmin^{23,29} predicts two types of internal disulphide bonding arrangements for the A domains of factor VIII. The disulphide structure proposed for the C domains of factor VIII is based on the proposition that the disulphide linkages form between the two cysteine residues found in each domain. The large B domain which separates the second and third A domains of factor VIII contains only four cysteine residues, but the presence of 19 asparagine-linked glycosylation sites suggests that this region is extensively modified by carbohydrate addition.

The activation of factor X by factor IX_a in conjunction with factor VIII is known to require calcium ions. Factor IX_a and factor X both contain γ-carboxyglutamic acid residues which are thought to be involved in calcium binding. The protein-bound calcium ions mediate the interaction of these proteins with the phospholipid surface. The homology of factor VIII with ceruloplasmin suggests the possible involvement of copper or other metal ions in the role of factor VIII in factor X activation. One possibility for the role of such metal ion involvement is suggested by the binding of lanthanide ions by γ-carboxyglutamic acid residues³⁰. It is interesting to speculate that the potential copper-binding ligands of factor VIII interact with a metal ion jointly bound by the γ-carboxyglutamate

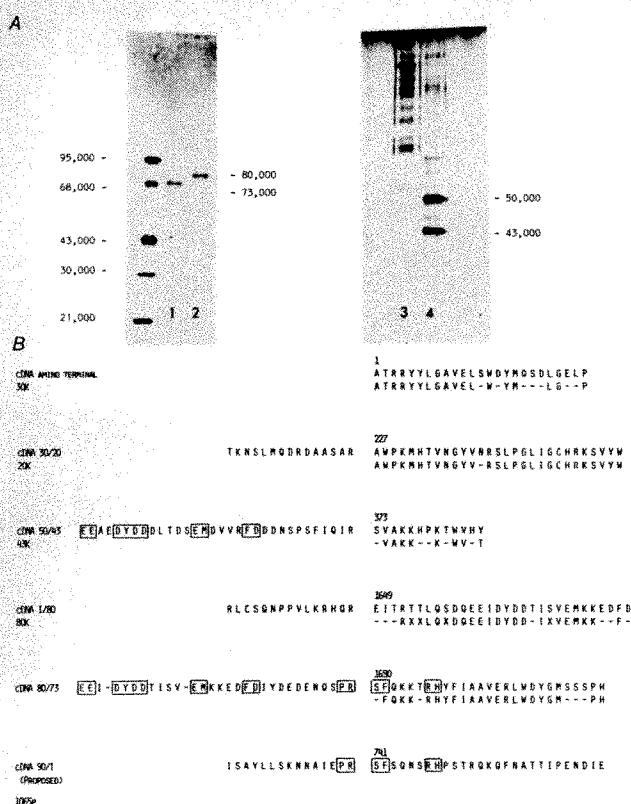
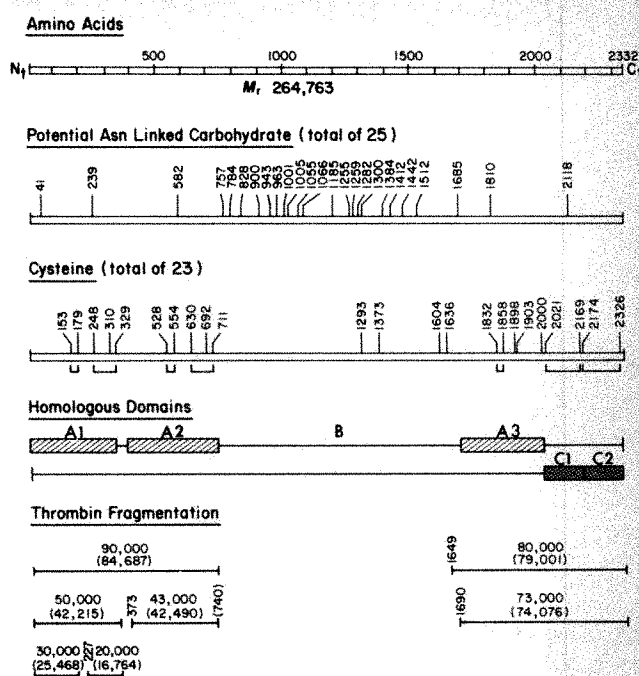


Fig. 5 A, Thrombin cleavage of separated factor VIII polypeptide fractions. B, Alignment of thrombin-generated amino termini with the deduced amino acid sequence¹⁹.

Methods: A, The factor VIII polypeptides were separated as described in legend to Fig. 2A. SDS was removed by dialysis of the fractions against 8 M urea solutions and urea removed by dialysis against 0.01 M Tris, pH 8.0. Thrombin was then added at weight ratios to a maximum of 1:20. The digestions were allowed to proceed at room temperature and the extent of cleavage was monitored by SDS-gel electrophoresis. Lanes 1 and 2 are the M_r 80,000 protein with and without thrombin, respectively; lanes 3 and 4 are the M_r 90,000–210,000 protein without and with thrombin, respectively. B, Thrombin digestion products were separated by preparative SDS-gel electrophoresis, electroeluted by the procedure of Hunkapiller *et al.*⁴¹ and analysed on a Beckman spinning cup sequencer⁴⁰. The M_r 80,000 protein was obtained by TSK separation as described for Fig. 2A. The thrombin cleavage site is indicated by a space in the cDNA deduced sequence. The number above the subsequent amino acid corresponds to the position of that residue in the linear sequence. The amino-terminal protein sequences for the various polypeptide chains are aligned under the translated gene sequence. —, Positions where no residue could be identified; X, positions where the wrong amino acid was determined. The relative molecular masses of the proteins separated are listed (30K is the amino terminus obtained for the gel-eluted polypeptide with a M_r of 30,000); solidi indicate cleavage products (for example, 50/43 indicates the cleavage which separates the M_r 50,000 and 43,000 species). Regions which share sequence homology are boxed.

residues of factors IX or X. In addition to copper transport and haemostasis, several enzymatic functions have been ascribed to ceruloplasmin, including ferroxidase activity, amino oxidase activity and superoxide dismutase activity^{31–35}. It will be important to determine whether any of these activities are associated with factor VIII.

The amino-terminal sequence of factor VIII thrombin fragments and the homology of factor VIII with ceruloplasmin provide insight into a functional purpose for the cleavages. Factor VIII isolated from plasma is usually degraded. In certain preparations, small amounts of a M_r 330,000 protein were observed when analysed on SDS-polyacrylamide gels run under non-reducing conditions; this protein was not observed when the factor VIII samples were reduced before electrophoresis. This reducible 330,000 may be a disulphide-linked, limited pro-



termini) from a single-chain circulating form. By analogy with factor V, the M_r 90,000 and 80,000 proteins would correspond to fragments D and E of factor V, respectively³⁴. These two fragments of factor V can be separated from the activation peptides and isolated as a functional two-subunit protein^{35,36}. Both subunits are required for factor V activity³⁵ and both may be required for factor VIII activity. A highly glycosylated intermediate region is cleaved from both proteins. Therefore, both factors V and VIII seem to be highly similar in structure, thrombin cleavage pattern and, presumably, function.

The studies described here provide a structural basis for defining the role of the diverse molecular forms of factor VIII in their interaction with other proteins of the coagulation cascade. The availability of complete factor VIII cDNA clones

capable of programming recombinant factor VIII synthesis in mammalian cell cultures¹⁹ will offer a unique opportunity to perform similar studies with the single-chain precursor molecule. The questions raised here concerning the relationship of processing events, structural domains and homology to ceruloplasmin with the biological function of factor VIII may be answered by studying structural changes introduced into the protein by modification of these cloned DNA sequences.

We acknowledge the support of Speywood Laboratories Ltd. We thank Dr John Bell, Alice Kleiss and William Henzel for laboratory assistance; Gary Hooper for his assistance in the coordination of the project between various groups and companies; and Robert A. Swanson and Dr David W. Martin Jr for their encouragement.

Received 13 August; accepted 27 September 1984.

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Molecular cloning of a cDNA encoding human antihaemophilic factor

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A complete copy of the mRNA sequences encoding human coagulation factor VIII:C has been cloned and expressed. The DNA sequence predicts a single chain precursor of 2,351 amino acids with a relative molecular mass (M_r) 267,039. The protein has an obvious domain structure, contains sequence repeats and is structurally related to factor V and ceruloplasmin.

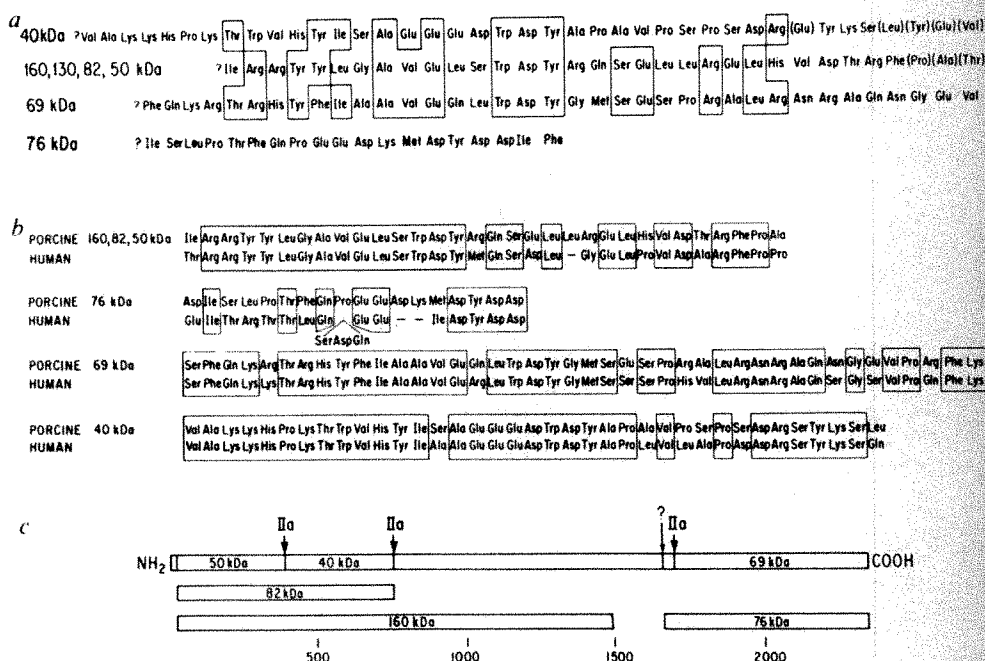
HAEMOPHILIA A is a bleeding disorder caused by deficiency or abnormality of a particular clotting protein, factor VIII:C¹ occurring in about 10-20 males in every 100,000. Afflicted individuals suffer episodes of uncontrolled bleeding and are treated currently with concentrates rich in factor VIII:C derived from human plasma. The available therapy, although reasonably effective, is very costly and is associated with a finite risk of infections. We report here significant progress in the use of recombinant DNA technology to provide pure human factor VIII:C as an alternative treatment for haemophiliacs.

Blood clotting begins with injury to a blood vessel. The damaged vessel wall causes adherence and accumulation of platelets activating the plasma proteins which initiate the coagulation process. Sequential activation, via specific proteolytic cleavages and conformational changes, of a series of proteins comprising the coagulation cascade eventually leads to deposi-

tion of insoluble fibrin which, together with aggregated platelets, curtails the escape of blood through the damaged vessel wall. Factor VIII:C is a large plasma glycoprotein that functions in the blood coagulation cascade as the cofactor for the factor IXa-dependent activation of factor X. It can be activated proteolytically by a variety of coagulation enzymes including thrombin².

In order to provide factor VIII:C for treatment of haemophiliacs we cloned a full-length cDNA. A major obstacle to the cloning effort was the large size of the protein, estimated to be at least M_r 250,000. Purification of factor VIII:C from plasma³ is made difficult by its low abundance, its extreme sensitivity to degradation by serum proteases and its tight association with polymeric forms of the more abundant protein, von Willebrand factor. Fass *et al.*⁴ have described a purification procedure for porcine factor VIII:C using monoclonal antibody

Fig. 1 *a, b*; N-terminal amino acid sequence analysis of bovine thrombin cleavage and natural cleavage fragments of porcine factor VIII:C. *c*, The positions of fragments in the factor VIII:C precursor in diagrammatic form. The molecular masses are the apparent porcine values deduced from SDS gel electrophoresis rather than those calculated from the human amino acid sequence. Map lengths are indicated in amino acid residues to correlate with Fig. 4. In *a*, N-terminal sequences were determined for the indicated fragments of porcine factor VIII:C. The first residue in each sequence could not be assigned and is indicated as ? In *b*, the porcine amino acid sequences are compared with the corresponding sequences in human factor VIII:C predicted from the nucleotide sequence of the cDNA clone (Fig. 4). Boxed areas, identity between sequences. Thrombin cleavage fragments of porcine factor VIII:C were purified by SDS polyacrylamide gel electrophoresis and located in the unfixed gel using a small portion of ^{125}I -labelled porcine factor VIII:C as a tracer. After electrophoretic elution from the gel matrix as described previously²⁸, the polypeptide sample was subjected to N-terminal amino acid sequence analysis using an Applied Biosystems gas-phase sequencer¹¹.



immunoaffinity chromatography, obtaining highly purified polypeptides of M_r 160,000, 130,000 and 76,000, similar in size to human factor VIII:C polypeptides⁵. Although it has been generally supposed that factor VIII:C, like its homologue factor V, is synthesized as a single chain macromolecular precursor which is then cleaved to yield the fragments found in purified preparations, the results presented here are the first conclusive demonstration that this supposition is correct⁶.

Additional obstacles to the isolation of a factor VIII:C cDNA clone were the lack of a cell line which made factor VIII:C and uncertainty about its site of synthesis *in vivo*⁷⁻⁹. Microsequencing of highly purified porcine factor VIII:C yielded sufficient amino acid sequence to construct oligonucleotide probes. We used these probes to identify a partial clone by direct screening of porcine genomic DNA libraries. The clone provided a probe for identification by cross-species homology of a corresponding human genomic clone. Using this probe, we determined that human liver is a source of factor VIII:C mRNA and obtained subsequently from such tissue the entire 9 kilobase (kb) cDNA sequence as a set of overlapping clones. Reconstruction of these clones into one continuous sequence within an expression vector provided a functional full-length cDNA able to express active protein after transfection into mammalian cells.

Porcine factor VIII:C

Treatment of highly purified porcine factor VIII:C with bovine thrombin (factor IIa) causes an initial activation of up to 60-fold⁴ concomitant with loss of the M_r ~160,000 polypeptide, transient appearance of a M_r 130,000 and the appearance of a new chain of M_r 82,000 (Fig. 1c). As the digestion proceeds, the M_r 76,000 polypeptide generates a fragment of M_r 69,000 and the M_r 82,000 polypeptide is cleaved, apparently to yield polypeptides of M_r ~50,000 and 40,000.

Our sequence analysis was performed using factor VIII:C purified from ~101 of porcine plasma collected from about ten hogs with precautions to minimize proteolysis during collection and subsequent processing¹⁰. We prepared 0.1–1.5 nmol of each of the M_r 160,000, 130,000 and 76,000 chains originally present in purified porcine factor VIII:C and the 82,000, 69,000,

50,000 and 40,000 thrombin cleavage fragments. N-terminal amino acid sequence analysis using the microsequencing procedures described previously¹¹ yielded the data presented in Fig. 1a, b. The M_r 160,000, 130,000, 82,000 and 50,000 chains have the same N-terminal amino acid sequence for at least thirty residues, whereas the 69,000 and 40,000 fragments have distinct N-terminal sequences. There is, however, striking homology between the 69,000, 40,000 and the 160,000 polypeptides and their fragments. The M_r 76,000 fragment shows no N-terminal sequence homology with the N-terminal sequences of any of the other fragments. Comparison of these sequences with partial sequence data for bovine factor V has led to a hypothetical model for the structural relationship between the putative single chain factor VIII:C and its cleavage products⁶ similar to that shown diagrammatically in Fig. 1c.

Genomic cloning

As the tissue source of mRNA for factor VIII:C was unknown, we tried the novel approach of directly screening genomic libraries with oligonucleotide mixtures to obtain a unique length of porcine factor VIII:C DNA. We used two probes, one a pool of long oligomers containing few sequences and the other a pool of shorter oligomers containing all possible sequences. We identified residues His8–Met22 of the M_r 69,000 chain (see Fig. 1) as containing the least codon ambiguity. The set of 45-mers described in the legend to Fig. 2 was selected on the basis of eukaryotic codon usage¹², the relative stability of G·T versus A·C mismatches¹³ and the infrequency of the dinucleotide CpG in eukaryotic genes. Jaye *et al.*¹⁴ used a similar strategy to generate long oligonucleotides for screening a cDNA library, but they did not attempt genomic screening. A set of 15-mers containing all possible sequences that could encode the Trp18–Met22 region of the M_r 69,000 polypeptide served as the secondary probe.

We constructed a genomic library from *Bam*HI digested female porcine DNA and screened it with the radiolabelled 45-mers under relatively nonstringent conditions (see Fig. 2 legend). Rescreening the library with the 15-mers yielded four clones out of 4×10^5 recombinants which hybridized strongly to

both the 45-mers and the 15-mers. The four bacteriophages were isolated and, on hybridization to additional oligonucleotides derived from the amino acid sequence of the M_r 69,000 polypeptide, one contained a 6.6 kilobase (kb) *Bam*HI fragment that hybridized to all probes (Fig. 2). Sequence analysis of DNA fragments subcloned into M13 confirmed that we had isolated a recombinant containing DNA, encoding a portion of the M_r 69,000 polypeptide. The 45-mer had 11% mismatch (5 of 45 nucleotide positions) but still hybridized strongly to the porcine genomic clone.

To obtain a human factor VIII:C genomic clone, the 6.6 kb *Bam*HI fragment from the porcine genomic clone was used to screen a human *Hae*III/*Alu*I library¹⁵. From 8×10^5 screened, a single recombinant phage containing an insert of about 16 kb was isolated (Fig. 2). The region of this clone containing sequence homologous to the porcine M_r 69,000 fragment, identified by hybridization to the 45-mers, was subcloned into M13 and its sequence determined. The sequence shows strong (>80%) homology to the porcine factor VIII:C sequence at both the nucleotide and derived amino acid level (see Fig. 1). Additionally, an exon/intron boundary occurs at precisely the same position in both porcine and human genomic DNAs.

To confirm further that we had cloned a segment of the human factor VIII:C gene, we demonstrated linkage to the X chromosome by comparing the Southern blot band intensities between human male diploid DNA and XXXXY DNA (human lymphoblastoid line GM 1202A from the NIGMS mutant cell repository). Several unique DNA fragments from the human genomic clone were used as probes. Each hybridized to a single human fragment which was appropriately overrepresented in the 4X DNA when compared with human albumin DNA internal controls.

The entire human factor VIII:C gene was isolated subsequently as overlapping clones spanning ~200 kb. We thus constructed a genomic library from the human XXXXY cell line and screened sequentially with unique probes from the ends of previously isolated recombinants. The factor VIII:C gene spans more than 180 kb; most of its exons are small (150–250 base pairs) with the exception of the 3.1 kb exon (Fig. 2) and the 1.9 kb 3'-most exon, which includes 1.8 kb of non-coding sequence.

Table 1 Expression of factor VIII:C cDNA in COS-7 cells

	Chromogenic activity (mU ml ⁻¹)	1 stage APTT assay (mU ml ⁻¹)
No DNA	<0.05	<<
pCVSVL	<0.05	<<
pCVSVL-VIII	7	10.0
+ thrombin	ND	50
+ α VIII IgG	<0.05	ND

The full-length cDNA clone was excised from pSP64-VIII with *Sal*I and inserted into the *Pst*I site in pCVSVL¹⁸ by addition to the vector of synthetic oligonucleotide adapters containing *Sal*I cohesive ends. The resulting plasmid containing the factor VIII:C cDNA in the correct orientation for transcription from the adenovirus major late promoter was designated pCVSVL-VIII. Plasmid DNA (8 μ g) was transfected into COS-1 monkey cells²³ in a 10 cm dish by the DEAE-dextran transfection protocol²⁴ with the addition of chloroquin treatment²⁵. The transfected cells were incubated for 36 h, then rinsed and fed with 4 ml serum-free media. Samples were taken for assay 24 h later. Factor VIII:C activity was determined by the Kabi Coatest factor VIII:C method modified to afford a sensitivity better than 0.05 mU ml⁻¹ and by the one-stage activated partial thromboplastin time (APTT) coagulation assay²⁶ using factor VIII:C-deficient plasma. For thrombin activation, samples were pretreated 1–10 min with 0.2 units ml⁻¹ thrombin at room temperature. The peak of activity (value shown) was reached after 4 min and then gradually declined. Inhibition with anti-factor VIII:C immunoglobulin was measured by the standard Bethesda protocol²⁷. Isolated immunoglobulin from serum of a patient with high titre factor VIII:C inhibitor was prepared by ion exchange and protein A Sepharose chromatography¹⁰. ND, not determined; <<, not detectable.

cDNA cloning

Our original human factor VIII:C genomic clone provided a probe with which to identify a useful tissue source for human factor VIII:C mRNA. A cell line secreting factor VIII:C is not available, but it had been reported that the protein was synthesized in liver and spleen^{7–9}. RNA blot analysis, initially with porcine material, confirmed the presence of very low mRNA levels in these tissues. Northern blot analysis of human liver mRNA, using a highly radioactive single-stranded probe of ~800 nucleotides of human factor VIII:C exon DNA (see Fig. 3 legend), demonstrated a single mRNA of ~9 kb (Fig. 3A, lanes a, b). A messenger of this size could encode a single-chain factor VIII:C precursor of M_r 250,000–300,000. In other experiments, the same size mRNA was detected in placenta but was absent from peripheral blood lymphocytes. All of these observations are consistent with the suggestion that factor VIII:C mRNA is synthesized by endothelial cells¹⁶.

We demonstrate in Fig. 3A (lane c) that the level of factor VIII:C mRNA in human liver is far lower than that of coagulation factor IX mRNA. Quantitative RNA dot blot hybridizations indicated that the amount of factor VIII:C mRNA was 20–40 times lower than factor IX mRNA. We had found previously that 1 in 5,000 recombinant phage in a liver cDNA library contains factor IX sequences. Thus we estimate that libraries of more than 200,000 recombinants would be required to isolate a single factor VIII:C clone.

Several large cDNA libraries were constructed in λ Charon 21A and GT10 (see Fig. 3 legend), using as a first strand primer either oligo(dT) or a unique 38-mer deduced from the sequence of the large human exon in Fig. 2. Ten factor VIII:C cDNA clones were isolated after screening ~2,000,000 recombinants

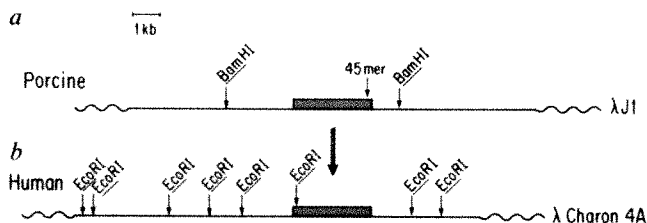


Fig. 2 a, Porcine and b, human factor VIII:C genomic clones. Homologous 3.1 kb exons are indicated by shaded boxes.

Methods: A porcine genomic library was constructed from *Bam*HI

digested female porcine liver DNA in the bacteriophage vector λ J1 (a derivative of L47.1) using established procedures. Recombinants (4×10^5) were screened using a modification of the procedure of Woo²⁹. A set of 45-mers containing the sequences 5'd (CATGCCATA^ATCCCACAGCTG^TTCCACAGCAGCAAT^AAGTAGTG) was synthesized on the Applied Biosystems Model 380A instrument. Filters were hybridized with 5×10^6 c.p.m. ml⁻¹ (0.5 pmole ml⁻¹) of ³²P-labelled 45-mers for 12 h at 45 °C in 5X SSC, 5X Denhardt's, 0.1% SDS, 100 μ g ml⁻¹ denatured salmon sperm DNA. Filters were washed at 50 °C in 5X SSC, 0.1% SDS, air dried and subjected to autoradiography using Kodak XAR film. After autoradiography, filters were denatured in 0.1M NaOH, neutralized and rehybridized with ³²P-labelled 15-mers, sequence 5'd (CATNCC^ATA^ATCCCA). Hybridization was performed as described above at 37 °C. Recombinants hybridizing to both oligomers were plaque purified and phage DNA prepared. Phage DNA prepared from one recombinant (PB34) contained a 6.6 kb *Bam*HI fragment which hybridized strongly to both sets of oligonucleotides in Southern blot analysis. The 6.6 kb *Bam*HI insert was purified from an agarose gel and ³²P-labelled by nick translation. Recombinants (8×10^5) from the human *Hae*III/*Alu*I genomic library of Lawn *et al.*¹⁵ were hybridized with 10^6 c.p.m. ml⁻¹ of the nick-translated porcine probe using the same conditions described for the 45-mer hybridization. A single recombinant phage, HH25, was thus isolated. *Sau*3A fragments of the insert from HH25 were subcloned into M13 and a phage which hybridized to the radio-labelled 45-mer was identified. The sequence of the *Sau*3A fragment in this subclone proved that HH25 contained DNA from the human factor VIII:C gene. The entire exon (shaded box) was subsequently sequenced. A 0.8 kb *Bam*HI fragment spanning the 3' exon/intron junction was subcloned as the probe to screen for a tissue source of factor VIII:C mRNA (see Fig. 3).

from the oligo(dT) libraries; 55 additional cDNA clones were obtained from among 1,000,000 recombinants from the 38-mer primed library. Four of the several clones characterized in detail are illustrated in Fig. 3B together spanning 9,000 base pairs (bp), sufficient to encode the entire mRNA detected by Northern

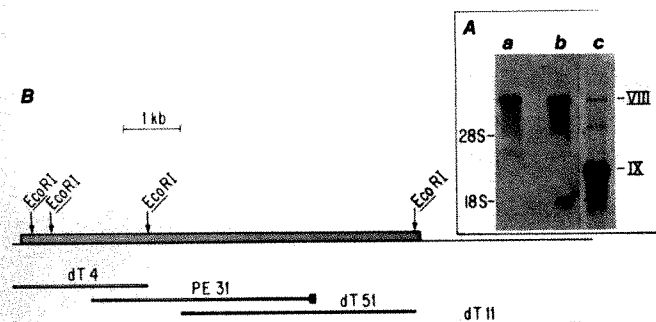


Fig. 3 A, Northern blot analysis to locate a tissue source for human factor VIII:C mRNA. Polyadenylated RNA (5 µg) extracted³⁰ from adult liver (lane a) or fetal liver (lanes b, c) was fractionated on an 0.8% agarose formaldehyde gel and transferred to nitrocellulose as described³¹. The filter in lanes a, b was hybridized with 5×10^6 c.p.m. of a ^{32} P-labelled factor VIII:C single-stranded probe prepared by primed synthesis from an M13 template which contained an 0.8 kb base *Bam*HI fragment of HH25 (see Fig. 2 legend)^{32,33}. The filter in lane c was probed in the same manner except 5×10^6 c.p.m. of a human factor IX probe of identical specific activity was used in addition. The exposure time for lanes a, b was longer than that for lane c and the RNA was from a different preparation. B, restriction map (*Eco*RI) of the human factor VIII:C cDNA sequence showing the single long open reading frame as a shaded box. An illustrative set of four overlapping bacteriophage λ clones are shown. Those designated dT4, dT51 and dT11 were from oligo(dT) primed λ Charon 21A libraries. Clone PE31 was from a library in λ GT10 primed with a unique 38-mer (solid rectangle). The cDNA sequence comprises 150 bases of 5'-untranslated region, an open reading frame of 7,053 bases and 1,806 bases of 3'-untranslated region.

Methods: Oligo(dT) primed double-stranded cDNA was synthesized from 10 µg polyadenylated fetal liver RNA as described³⁴. 200 pmol of a unique complementary 38-mer (nucleotides 5,308–5,345) and 10 µg polyadenylated fetal liver mRNA were denatured in 10 mM CH_3HgOH . The reaction was made 140 mM in 2-mercaptoethanol, 700 mM KCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8.3 at 42°C), 1 unit μl^{-1} of RNasin (Promega-Biotec) and incubated at 50°C for 2 min and at 42°C for 2 min. First and second strand synthesis was then performed as for the oligo(dT) primed cDNA³⁴. The second strand reaction was terminated by the addition of EDTA to 20 mM. The *Eco*RI sites were methylated, after the addition of S-adenosylmethionine to 50 µM and 40 units of *Eco*RI methylase, by incubation at 37°C for 1 h. These reactions were terminated by phenol-chloroform extraction and chromatographed using Sephadex G-50 equilibrated in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl. DNA in the excluded volume was pooled and precipitated with ethanol. The double-stranded cDNA was blunted in 50 µl containing 50 mM Tris pH 8.3, 10 mM MgCl_2 , 10 mM 2-mercaptoethanol, 50 mM NaCl, 50 µM of deoxynucleotide triphosphate, 100 µg ml^{-1} ovalbumin and 5 units of T4 polymerase (PL Biochemicals). The reaction was incubated at 37°C for 30 min and then terminated by phenol-chloroform extraction. Nucleic acids were recovered by ethanol precipitation with 2 M ammonium acetate. Kinased *Eco*RI linkers (NE Biolabs; 300 ng) were then added to the blunted double stranded cDNA by ligation overnight at 16°C in a final volume of 30 µl. The reaction was diluted to 200 µl in *Eco*RI digestion buffer and digested with 300 units of *Eco*RI (NE Biolabs) for 2 h at 37°C. EDTA to 15 mM was added and the reaction extracted with phenol-chloroform and chromatographed over Sepharose CL-4B (Pharmacia) equilibrated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl. cDNA in the void volume was collected and precipitated by ethanol in the presence of 10 µg yeast tRNA carrier. cDNA was redissolved in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and ligated to *Eco*RI cleaved, phosphorylated, λ Charon 21A DNA for the oligo(dT) primed cDNA and to λ GT10 DNA for the primer extended cDNA. The libraries were packaged, plated and initially screened using a unique ^{32} P-labelled 53-mer (nucleotides 5,158–5,210) permitting the isolation of both PE31 and dT51. Recombinant dT4 was identified with a ^{32} P-labelled pool of 64 56-mers containing the sequences: 5'd(TCCCAATCCTCTCTTC¹GC²GA³ATATAGTG⁴ACCCA⁵GTCTT⁶GGGTGCTTCTT) (using the same criteria as was used for the construction of the 45-mer pools) made to residues (Lys3-Asp21) of the *M*_{40,000} polypeptide fragment of porcine VIII:C. Recombinant dT11 was isolated using a nick translated 3' end *Pst*I/*Eco*RI fragment (nucleotides 5,902–7,127) of dT51. Numerous other cDNA clones were isolated by similar methods; some were also characterised in detail, sequenced and used in the construction of the full-length cDNA clone. Selected cDNA and genomic exon fragments were ligated to one another two at a time, subcloned and ultimately inserted in the *Sal*I site of pSP64 after the addition of a synthetic *Sal*I site at nucleotide 145. The resulting recombinant, pSP64-VIII, contains the factor VIII:C cDNA from just before the initiator ATG (at nucleotide 150–152) to nucleotide 7,422 (some 200 bp downstream from the terminator at nucleotide 7,201).

blot analysis. (The unique 9,009 bp cDNA sequence we obtained after sequencing numerous overlapping fragments of cDNA and genomic DNA has been deposited in the NIH US Nucleic Acid Sequence Databank and is available through GENBANK.) The sequence comprises a 5'-noncoding region of 150 nucleotides, a single long open reading frame of 7,053 nucleotides (2,351 codons) and 1,806 nucleotides of the 3'-noncoding region. The translated sequence, in a single-letter amino acid code, is presented as Fig. 4.

A full-length cDNA clone was assembled (see Fig. 3 legend) and inserted into the *Sal*I site of the polylinker in plasmid pSP64 (Promega Biotec). We thus obtained a recombinant, pSP64-VIII, with a single continuous factor VIII:C coding sequence that could be readily excised by *Sal*I digestion for insertion into a variety of expression vectors.

Expression and structure

To confirm that the factor VIII:C cDNA indeed encodes active factor VIII, we introduced the full-length cDNA into the mammalian expression vector pCVSVL^{17,18}. Plasmid pCVSVL-VIII contains the cDNA in the proper orientation to be expressed from the adenovirus major late promoter. Medium collected 60 h after transfection of COS-1 cells with this plasmid contained readily detectable levels of factor VIII:C activity (Table 1). No activity was detected in controls. The activity was inhibited by antibody to human factor VIII:C and was activated fivefold by thrombin.

The sequence of the cDNA clone comprises 2,351 amino acids from the initiator Met to the terminating Tyr residue and begins with a secretory leader peptide sequence of 19 amino acids. Regions of homology with the previously determined amino acid sequences of porcine factor VIII:C fragments can be located (Fig. 1), confirming the model for the structure of porcine factor VIII:C of Fass *et al.*⁶ and predicting the mature N-terminus and thrombin cleavage sites of the human molecule.

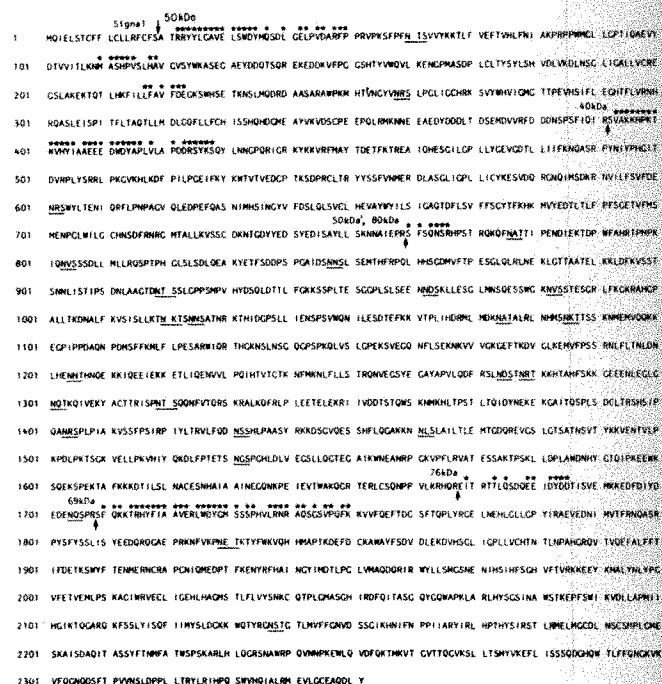


Fig. 4 Amino acid sequence of the human factor VIII:C precursor, predicted from the sequences of cDNA and genomic clones. Arrows indicate (1) a presumed signal peptidase cleavage site at residues 19–20 (Ser–Ala); (2) thrombin cleavage sites at residues 391–392, 759–760 and 1,708–1,709 (all Arg–Ser); (3) a possible thrombin cleavage site at residues 1,667–1,668 (Arg–Glu). Before each cleavage site the apparent *M_r* of the corresponding porcine factor VIII:C fragment C-terminal to the cleavage site⁶ is given. *, Amino acid residue identity with porcine factor VIII:C amino acid sequence determined from a variety of proteolytic and chemical cleavage fragments. Potential N-glycosylation sequences (Asn–X–Ser/Thr) are underlined.

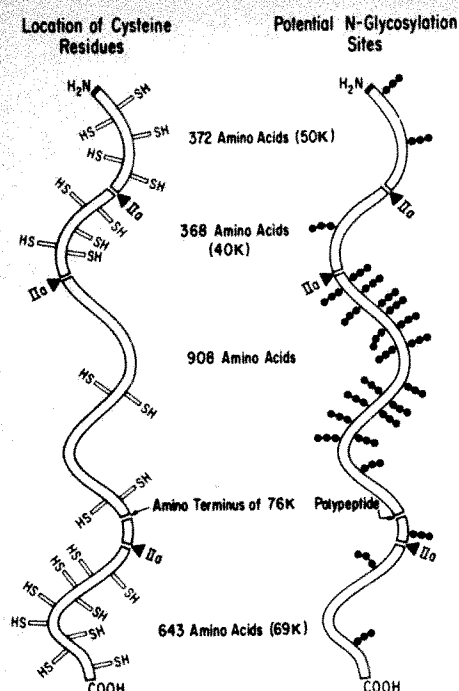


Fig. 5 Structural features of human factor VIII:C, showing schematically the location of cysteine residues and potential N-glycosylation sites (Asn-X-Ser/Thr) with respect to thrombin cleavage domains. The number of amino acid residues in each domain is given as well as the apparent molecular weights of the corresponding regions of porcine VIII:C.

The location of cysteine residues and potential N-glycosylation sites (Asn-X-Ser/Thr) is shown schematically in Fig. 5. Their exact numerical position within the human factor VIII:C precursor molecule is shown in Fig. 4. The cysteine residues appear to be clustered in the M_r 50,000, 40,000 and 69,000 regions. There are no disulphide bridges between these regions in porcine factor VIII:C, where the same pattern of polypeptides is observed on SDS gels in either reducing or non-reducing conditions.

In contrast to the location of cysteine residues, many of the potential N-glycosylation sites appear between the M_r 40,000 and 69,000 regions (Fig. 5). Distinct bands corresponding to this domain of 908 amino acids, or cleavage fragments derived from it, cannot be readily identified on silver stained SDS gels. We have obtained amino acid sequence data from minor components in purified porcine factor VIII:C preparations which are present in the M_r 80,000 and 50,000 regions of SDS gels. The N-terminal sequences of these polypeptides are identical; furthermore, a similar amino acid sequence is found in the predicted human factor VIII:C precursor amino acid sequence shown in Fig. 4 (residues 760-766).

A striking feature of human factor VIII:C is the presence of repeated blocks of amino acid homology (Fig. 6); the three most extensively repeating patterns of homology are within the M_r 50,000 and 69,000 fragments (blocks A, B, C, D, E and A', B', C', D', E'); within the M_r 50,000 and 40,000 fragments (blocks A, F, G, H, I and A', F', G', H', I') and a M_r 69,000 fragment internal repetition (blocks J, K, L and J', K', L'). Another set of repeat blocks (A', M and A'', M') is suggestive of a vestige of a M_r 40,000/69,000 duplication. Not only is there a strong M to M' homology, but also the A to M spacing is very similar in both regions. A block of amino acids (N) in the 40,000 region is repeated (N') in the region between the M_r 40,000 and 76,000 regions.

We analysed the precursor human factor VIII:C amino acid sequence by the method of Hopp and Woods¹⁹ for the determination of relative hydrophobicity. As observed for other secreted proteins, the factor VIII:C precursor contains a hydrophobic

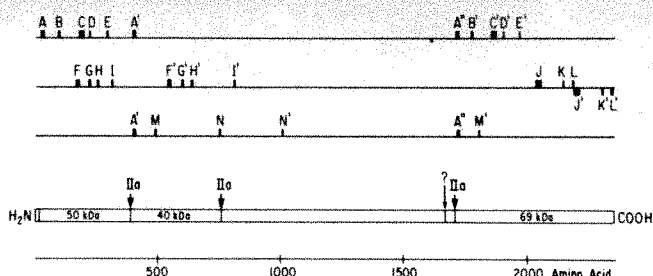


Fig. 6 Amino acid sequence homology within human factor VIII:C. A computer search for internal amino acid sequence homology was performed using the PEP program in the IntelliGenetics software system. Blocks of homology are labelled alphabetically on the linear maps, with their location and relative size indicated. Matching blocks of homology bear the same alphabetical symbol plus a prime or double prime. The actual amino acid sequences within these blocks and their numerical position with the entire precursor molecule can be located in Fig. 4. A(residues 21-35); A'(400-414); A''(1,714-1,728); B(89-101); B'(1,776-1,788); C(177-198); C'(1,856-1,877); D(211-227); D'(1,902-1,908); E(292-298); E'(1,979-1,985); F(164-179); F'(539-554); G(217-227); G'(594-604); H(250-261); H'(633-644); I(318-324); I'(809-815); J(2,038-2,059); J'(2,191-2,212); K(2,144-2,151); K'(2,301-2,308); L(2,181-2,188); L'(2,338-2,345); M(486-492); M'(1,796-1,802); N(749-755); N'(1,002-1,008).

leader sequence (residues 1-19). Two other extensive regions of hydrophobicity are also predicted by this analysis; first, 27 amino acids from Ser308 to His334 and second, from Ser630 to Phe677. The hydrophobic nature of these regions is substantiated by the method of Kyte and Doolittle²⁰. The role of these hydrophobic regions in factor VIII:C secretion and/or activity is unknown.

Homology with other plasma proteins

We have reported previously²¹ amino acid sequence homologies among certain regions in factor VIII:C, factor V, and ceruloplasmin. These homologies were identified by comparison of the N-terminal amino acid sequences of thrombin cleavage fragments of bovine factor V and porcine factor VIII:C with amino acid sequence deduced from a genomic clone of porcine factor VIII:C and with the published amino acid sequence of human ceruloplasmin²². The deduced amino acid sequence of human factor VIII:C described here (Fig. 4) contains regions of homology with the known amino acid sequences of bovine factor V and extensive homology with the entire human ceruloplasmin molecule, supporting the suggestion that these three proteins are related evolutionarily. Note the homology between blocks A, A' and A'' of human factor VIII:C (Fig. 6) and residues 2-16, 353-366 and 712-726 respectively of human ceruloplasmin²², as these homology blocks apparently mark the boundary of a primordial gene encoding approximately 350 amino acids. Similarly, block A is homologous with residues 3-17 of the M_r 94,000 amino terminal thrombin cleavage fragment of bovine factor V and block A'' is homologous with residues 6-20 of the C-terminal M_r 74,000 thrombin cleavage fragment of factor V²¹. At present, the significance of repeating homologous units in factor VIII:C, factor V and ceruloplasmin is unclear, but they presumably reflect duplications and triplications of some smaller ancestral gene.

We thank Louise Wasley and John Brown for technical assistance; Ed Fritsch and Steve Clark for support and helpful discussions; Joyce Lauer and Katherine Smith for help with the manuscript and Martha Richardson, Marybeth Erker and Cathy Beechinor for word processing. Finally, we thank both Robert Kamen and Gabriel Schmergel for their encouragement throughout the project. This work was supported by Baxter Travenol Inc., the Mayo Foundation and grant HL-17430 to D.N.F.

Received 31 August; accepted 2 October 1984.

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LETTERS TO NATURE

Cosmic-ray emission from Cygnus X-3

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Recent observations^{1,2} of the X-ray binary source Cygnus X-3 have revealed a flux of ultra-high energy γ rays. Other observations and subsequent models suggest that Cyg X-3 may be a dominant source of galactic high-energy cosmic rays. Here I investigate whether it is possible to escape this conclusion with a model in which most of the energy of accelerated particles at the source is emitted as curvature radiation (that is, radiation emitted by particles travelling on curved paths in a magnetic field so strong that synchrotron radiation is suppressed). I conclude that such a model is not consistent with the observations.

The ultra-high energy γ -ray flux^{1,2} at 10^{15} – 10^{16} eV from the X-ray binary source Cygnus X-3 has attracted much attention. If the output is in the form of a rotating beam or an interception of a particle beam by a producing medium in the line-of-sight, the estimated³ emission in the energy range is 6×10^{36} erg s⁻¹. The flux at 10^{11} – 10^{13} eV seen by atmospheric Čerenkov groups⁴⁻⁷ also contributes $\sim 10^{37}$ erg s⁻¹, again assuming a beamed emission. This γ -ray component may be significant at high energies in forming cosmic-ray anisotropies⁸. Because of the difficulty in explaining the ultra-high energy flux in terms of electron emission^{2,9,10}, a proton or ion mechanism, probably involving nuclear collisions and production of π^0 -mesons¹¹, seems necessary. Using nuclear interaction models of the source, it has been shown^{12,13} that more than 10^{40} erg s⁻¹ of high-energy cosmic-ray particles are emitted. The calculation of the production and subsequent absorption of γ rays in a uniform medium gives a maximum efficiency of only $\sim 3\%$ for the conversion of a nuclear spectrum into a γ -ray spectrum of comparable exponent. Interstellar absorption, for the accepted distance of 11.4 kpc (ref. 3), raises the observed flux to $\sim 3 \times 10^{37}$ erg s⁻¹ in the region 10^{15} – 10^{16} eV. These are time-averaged fluxes. In a directed effect, in which an isotropic flux of protons or ions strikes an emitting/absorbing medium¹³ and if the observed duty cycle is 0.1 (in ref. 2 it seems to be as low as 0.025), then we arrive at a heavy-particle emission of $\geq 10^{40}$ erg s⁻¹. However, the total cosmic-ray energy injection rate required for the whole Galaxy is thought^{14,15} to be only 10^{40} – 10^{41} erg s⁻¹. Thus a single Cyg X-3 type source could supply the entire cosmic-ray demand up to particle energies of $\sim 10^{16}$ eV.

Hillas¹³ holds that Cyg X-3 can comfortably supply the ultra-high energy galactic cosmic rays in the energy range 10^{15} – 10^{18} eV, but probably does not contribute substantially to the lower energy flux. Does the observed γ -ray flux necessarily imply such large proton fluxes, or are there are mechanisms which could supply the photon component more economically? One possible mechanism, curvature radiation¹⁶, is considered here because it has a very efficient transfer ratio between emitting particles and emitted photons, and because it operates with heavy particles as well as electrons. For the 10^{15} – 10^{16} eV band,

protons or ions are required, but electrons might produce the 10^{11} – 10^{13} eV component. For the 10^{13} – 10^{15} eV interval, for which there is some experimental evidence of emission¹⁷, there could be a combined effect, and indeed the phase of the 4.8-h period emission seems to change between 10^{12} and 10^{15} eV. For either proton or electron emission, however, there is a serious γ -ray absorption problem, arising from pair production in the magnetic field of the pulsar¹⁸. Ignoring this we can calculate, in cgs units, the required power output. For a single particle of unit charge, whether electron or proton, the power output in curvature radiation is:

$$W = 2e^2 c \gamma^4 / 3r^2 \quad (1)$$

With a characteristic γ -ray energy

$$h\nu = 2\pi\hbar c \gamma^3 / r \quad (2)$$

where r is the radius of the orbit, $\gamma = E/mc^2$ for either a proton or an electron, where E is the energy of the proton or electron.

For the 10^{15} -eV γ rays we take $r = 10^8$ cm, for a field line extending from the pulsar pole to the velocity of light cylinder. The frequency $\nu = 10^{30}$ Hz, and from equation (2) we have $\gamma^3 = 3 \times 10^{27}$ or $\gamma = 10^9$. For a proton this gives a particle energy of 10^{18} eV. For orbit radii $< 10^8$ cm, W would be large, resulting in a rapid energy loss, so that the process would not work.

Acceleration to this energy may be electrostatic¹⁰. The field ϵ has a maximum value^{14,15} given by $\epsilon = 10^{-14.2} B r^{3/2} P^{-3/2}$ V cm⁻¹ where B is the magnetic field and P the period; at 10^{12} G and 3 ms respectively we obtain a field of $\sim 2 \times 10^{13}$ V cm⁻¹ near the pole of the pulsar. The distance to the velocity-of-light cylinder is only 10^7 cm, but in the absence of short-circuiting effects, it is not difficult to obtain potentials in the region of 10^{20} eV. An alternative possibility is the mechanism of Gunn and Ostriker¹⁹.

The energy output in curvature radiation from a proton of 10^{18} eV is given by equation (1):

$$W = \frac{2}{3} \times \frac{25 \times 10^{-20} \times 3 \times 10^{10} \times 10^{36}}{10^{16}} \\ = 10^{12} \text{ erg s}^{-1} \text{ or } 30 \text{ erg cm}^{-1}$$

This is essentially equal to the electrostatic energy gained, so that there will be equilibrium between emission and acceleration. For a flight distance 10^7 cm to the velocity-of-light cylinder, we have an emission of 3×10^8 erg at a constant particle energy of 10^{18} eV. To produce 3×10^{37} erg s⁻¹ of γ rays at 10^{15} eV we require 10^{29} particles s⁻¹, each with 10^{18} eV or 10^6 erg, making a total energy flux of $\sim 10^{35}$ erg s⁻¹. We assume that all particles escape on reaching the velocity-of-light cylinder.

Therefore, the proton flux produced might be less by more than two orders of magnitude than the γ -ray flux, and five orders of magnitude less than the 10^{40} erg s⁻¹ required by the nuclear interaction model. A similar calculation for electrons having characteristic energies of 10^{14} eV results in a comparable energy requirement, about 10^{34} erg s⁻¹ of 10^{14} eV electrons producing

$10^{37} \text{ erg s}^{-1}$ of γ rays at 10^{12} eV . The assumptions made about magnetic and electrostatic fields are, of course, optimistic, but a more accurate calculation would probably not differ from the above by more than two orders of magnitude.

There is, however, a severe difficulty in extracting the γ rays from the system without pair production in the magnetic field. There will probably also be considerable but lesser geometrical difficulties in producing the characteristic one or two narrow γ -ray pulses in the 4.8-cycle.

The condition for pair production is $B_{\perp} E_{\gamma} > 10^{18.6}$, with B in gauss and photon energy in electron volts¹⁶. For $E_{\gamma} = 10^{15} \text{ eV}$ the transverse components of B must be $\leq 10^4 \text{ G}$. With a surface field of 10^{12} G and an orbit radius of 10^8 cm this cannot be achieved, and escape would be possible, if at all, only from a position very close to the velocity-of-light cylinder. The difficulties are not quite so severe for 10^{12} eV γ rays. The field at 10^7 cm would be $\leq 10^9 \text{ G}$, and the angle between the photon trajectory and field line could be $< 10^{-2}$, if the orbit radius were increased to 10^9 – 10^{10} cm . This, however, involves increases in electron

energies and fluxes to a point where $\sim 10^{38}$ – $10^{39} \text{ erg s}^{-1}$ are needed to produce $10^{37} \text{ erg s}^{-1}$ of 10^{12} eV γ rays. For protons and 10^{15} eV γ rays it is difficult to see how the model can work without requiring particle fluxes approaching the $10^{40} \text{ erg s}^{-1}$ demanded by the nuclear interaction model.

Another model involves a black hole²⁰. It is not yet clear whether γ rays of 10^{15} eV can be produced in this. Alternatively, emission by quantum synchrotron radiation from electrons in high magnetic fields is possible, but this seems to demand very artificial geometries: electrostatic acceleration of the particles in a region essentially free from magnetic field, followed by almost discontinuous injection into the region of a high field. Otherwise, dissipation of particle energy by low-energy synchrotron emission seems inevitable. Similar models with protons as the radiating particles seem to require unrealistically high particle energies.

The nuclear interaction model needs to be developed to see whether it can produce the experimentally-required spectra and nuclear constitution observed in cosmic rays.

Received 8 May; accepted 10 September 1984.

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A disk origin for superhumps in Ursae Majoris variables

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As cataclysmic variables, SU Ursae Majoris stars^{1,2} are short-period binary systems consisting of a red dwarf star that fills its Roche-lobe and spills material to an accretion disk around a white dwarf. SU UMa systems undergo outbursts (episodes of increased mass transfer in the disk) of two distinct types: normal outbursts that last a few days, and superoutbursts that last up to 2 weeks. Superhumps are periodic $\sim 30\%$ brightness variations of unknown origin seen only during the superoutbursts. The superhump period is several per cent longer than the binary orbital period. Both the accretion disk^{3,4} and the red dwarf⁵ have been proposed as possible sites for the superhump. Although superhumps occur in both eclipsing and non-eclipsing systems, observations of the eclipsing systems Z Cha and OY Car may help to discriminate between these two classes of models. Whitehurst *et al.*⁶ have shown that the superhumps in Z Cha are not deeply eclipsed, and argue that superhumps therefore cannot originate in the accretion disk. In their starspot model, superhumps arise in a bright active region located in the equatorial belt of the red dwarf. By contrast, the light curve synthesis results reported here show explicitly that disk models produce a shallow partial eclipse of the superhump light, compatible with the eclipse depth measurements of Whitehurst *et al.*, provided the superhump source is not concentrated into a small region of the disk. The starspot model is shown to be inconsistent with the observed shapes of superhumps unless radiation emerges from the starspot in a narrow ($\leq 35^\circ$) fan beam with its long axis in the direction of stellar latitude.

Z Cha is depicted in Fig. 1 as viewed from the Earth at three different orbital phases (ϕ_{orb}). Deep eclipses of the optical light from the system are seen at $\phi_{\text{orb}} = 0.0$ (Fig. 1c), when the red dwarf occults the accretion disk, but secondary eclipses are not seen at $\phi_{\text{orb}} = 0.5$ (Fig. 1a), when the disk occults one hemisphere of the red dwarf. Successive eclipses of the accretion disk occur progressively earlier in the superhump cycle, because the super-

hump period is a few per cent longer than the 107-min orbital period. Eclipse light curves thus probe changes in the structure of the accretion disk during the superhump cycle, and can be used to establish the site of the superhump source.

To determine whether the superhumps in Z Cha are eclipsed, Whitehurst *et al.* measured the brightness of Z Cha at moments of minimum light, when much of the accretion disk is occulted by the red dwarf (Fig. 1c). To correct approximately for secular changes in the brightness of Z Cha during the superoutburst, the fluxes at mid-eclipse are first divided by fluxes at nearby phases that are not affected by either the eclipse or the superhump. Complete coverage of the superhump cycle is achieved by combining measurements from three different superoutbursts. Whitehurst *et al.* found that the normalized mid-eclipse fluxes increased by $\sim 25\%$ for eclipses that occur near the superhump peak, and were roughly consistent at all superhump phases with the expected fluxes for an uneclipsed superhump. Thus the superhump source in Z Cha is not deeply eclipsed.

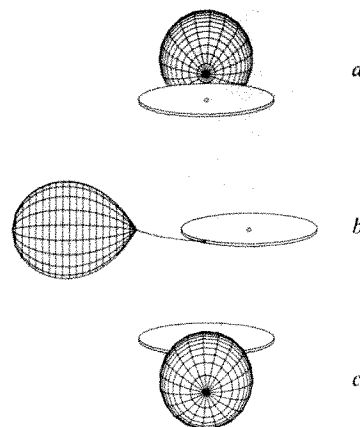


Fig. 1 Schematic views of the cataclysmic variable star Z Cha, at three different binary phases. The red dwarf star is severely distorted by tidal stresses induced by its white dwarf companion. An accretion disk surrounding the white dwarf is fed gas in a stream from the red dwarf originating at the inner lagrangian point. One hemisphere of the red dwarf is largely eclipsed by the disk (a), and a large area of the disk remains visible at mid-eclipse (c).

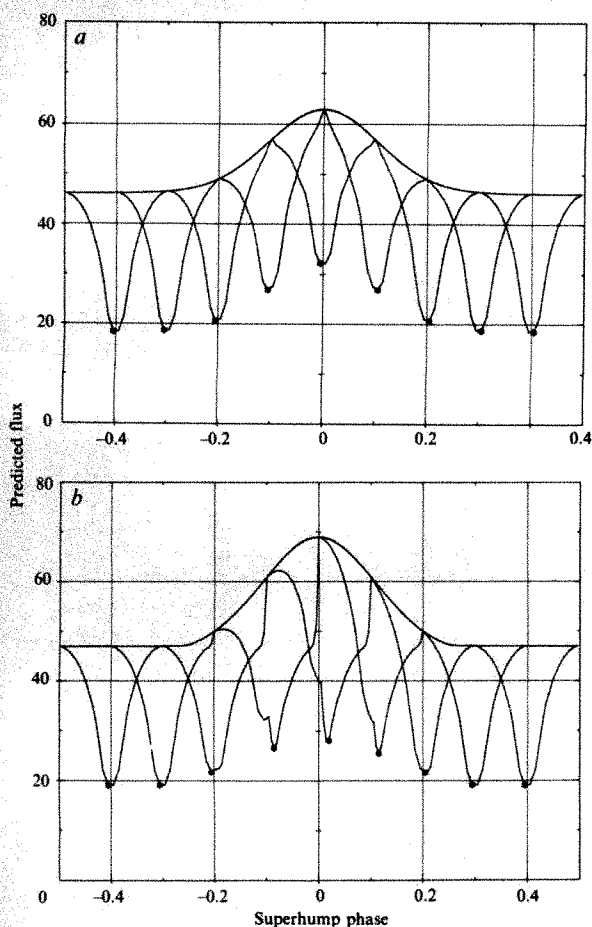


Fig. 2 Synthetic light curves for a disk model of superhumps. The upper envelope, giving the superhump light curve in the absence of eclipses, is produced in the model by modulating the surface brightness in the outer annulus of the accretion disk. Eclipses of the disk at different superhump phases are shown. *a*, A complete full annulus at the outer rim of the disk is modulated. The eclipse shapes in this case remain similar throughout the superhump. *b*, The modulation is confined to a single point on the outer rim of the disk. The distorted eclipses found in this case are not observed, ruling out a compact region on the disk as the site of superhumps. In both cases, the mid-eclipse flux is larger during the superhump because the accretion disk is only partially eclipsed.

The absence of deep eclipses of the superhump light in Z Cha does not preclude a disk origin for superhumps. Although the bright central region of the accretion disk is eclipsed, a large fraction of the disk surface remains visible at minimum light (Fig. 1c). A superhump source that is broadly distributed on the face of the accretion disk therefore undergoes a partial eclipse which may be quite shallow, for example, if the superhump light is distributed around the outer annulus of the disk. Thus, the eclipse depth measurements of Whitehurst *et al.* are compatible with partially-eclipsed superhumps produced in the accretion disk. Smak⁶ independently reaches this same conclusion.

The viability of a disk origin for superhumps is explicitly demonstrated by the synthetic eclipse light curves shown in Fig. 2. Here the uneclipsed superhump light curve is the upper envelope of nine individual eclipse light curves, each centred at a different superhump phase. Fluxes at minimum light, marked by stars in Fig. 2, increase during the superhump in the manner required by the eclipse depth measurements of Whitehurst *et al.* The times of minimum light are slightly advanced during the rise of the superhump, and delayed during the decline. Thus the eclipse phase shift discussed by Whitehurst *et al.* in the context of the starspot model is also produced by a disk model.

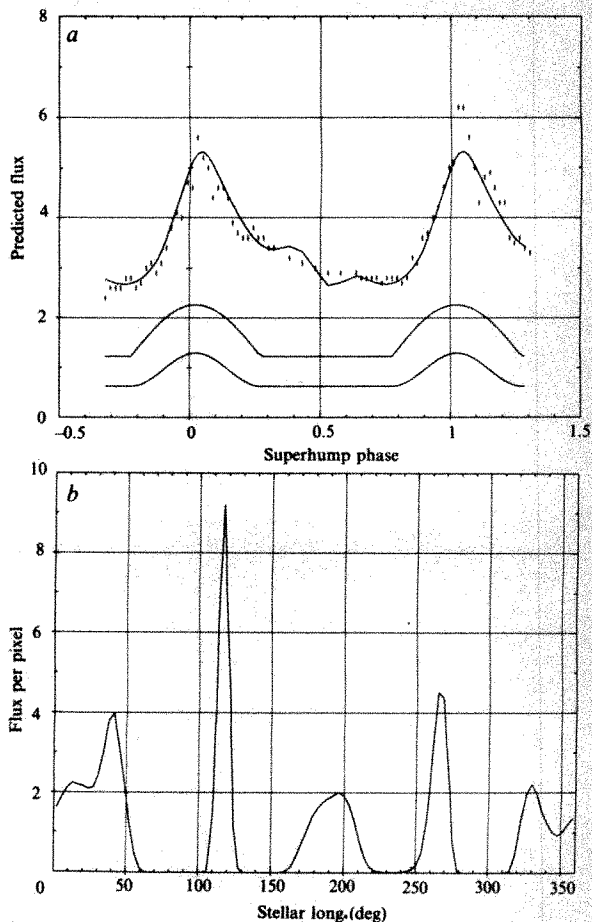


Fig. 3 The synthetic light curve for a rotating spherical star with a strip of non-uniform brightness around the stellar equator is compared in *a* with an observed superhump light curve of Z Cha. The longitudinal brightness distribution of the fitted model is given in *b*. The light curve peak produced by this starspot model is always broader than the observed superhump, because the light curves of point sources on the stellar equator (undarkened and fully limb-darkened cases are shown) are already too wide. Flux is given in arbitrary units.

The qualitative behaviour illustrated by this simulation of superhumps from the accretion disk is not sensitive to errors in the assumed geometry of the Z Cha system, to specific details of the accretion disk model, or to the assumed spatial distribution and temporal development of the superhump light, provided only that the superhump source is broadly distributed on the accretion disk. The eclipse geometry (Fig. 1) is the same as that assumed by Whitehurst *et al.* The accretion disk brightness distribution is a steady-state black-body accretion disk model that roughly reproduces the shape and depth of the observed eclipses of Z Cha during superoutburst. The red dwarf is assumed to contribute no light.

The uneclipsed shape of the superhump can be specified arbitrarily and is therefore not an important feature of the simulation. The superhumps in Fig. 2 are produced by modulating the temperature (T) of the outer annulus of the accretion disk according to $T \propto \exp(-2 \cos \phi_{sh})$, where ϕ_{sh} is the superhump phase. This specific form is motivated by the model of Papaloizou and Pringle³ in which mass transfer from the red dwarf varies exponentially with small changes in the binary separation caused by a slightly eccentric orbit, while the discrepant orbital and superhump periods are explained as a precession of the line of apsides.

If the superhump light curve in the absence of eclipses is known, the observed eclipse light curves give detailed information about the spatial distribution of the superhump source on the accretion disk. The eclipse shapes are not strongly affected

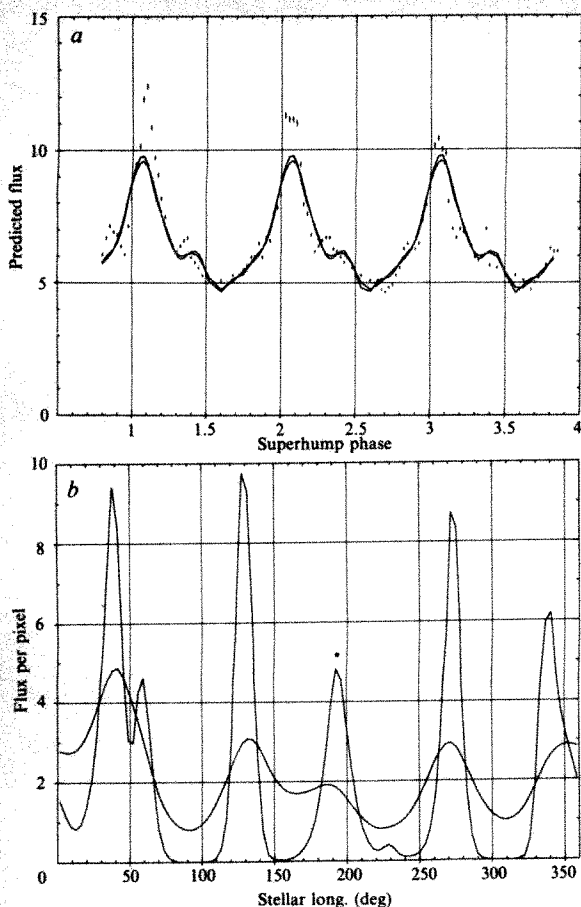


Fig. 4 The starspot model fails to produce the sharply-peaked superhumps observed in OY Car. Two models with different longitude resolution are shown. *a* and *b* are as in Fig. 3.

by the superhump if, as in Fig. 2*a*, the superhump light is uniformly distributed around the outer annulus of the disk. A superhump source confined to a compact region on the accretion disk produces highly distorted eclipses (Fig. 2*b*). Because such distortions are not observed, the main source of superhump light cannot be the white dwarf, the compact hotspot where the gas stream from the red dwarf feeds matter to the accretion disk, or any other compact region on the accretion disk. More detailed modelling of the eclipse observations will refine our knowledge of the spatial distribution of the superhump source, but this programme is complicated by the need to disentangle changes in the eclipse shapes from changes in the shape of the superhump. The slow march of the eclipses across the superhump light curve occurs on a time scale comparable with that for secular evolution of the superhump light curve itself.

In the starspot model proposed by Whitehurst *et al.*, superhumps are caused by a bright active region on the red dwarf. The discrepancy between the orbital and superhump periods is interpreted as a slight prograde rotation of the red dwarf in the frame of the orbital motion of the binary. The bright starspot must lie near the stellar equator to avoid occultation at $\phi_{\text{orb}} = 0.5$ by the accretion disk, because secondary eclipses are not observed.

Synthetic light curves for the starspot model are compared in Figs 3 and 4 with the observed superhump light curves of OY Car and Z Cha. Observations of OY Car by Kzreminski⁷ are shown in Fig. 3*a*. Warner's observations of Z Cha, as given by Cook⁸, are shown in Fig. 4*a* (also Fig. 2 of Whitehurst *et al.*). The light curves show typical examples of the large superhumps that develop a few days after the start of a superoutburst. A constant, equal to the depth of the eclipse, is subtracted from the original data to remove the light of the accretion disk. The amplitude of the brightness variation to be accounted for by the rotating

spotted star is thereby underestimated, as part of the light visible at mid-eclipse is, in fact, from the accretion disk. The accretion disk eclipses, which occurred at $\phi_{\text{sh}} \approx 0.5$ for Z Cha and $\phi_{\text{sh}} \approx 0.6$ for OY Car, are removed by interpolating observations before and after the eclipse.

Light curves for the starspot model are computed by summing contributions from an array of discrete sources distributed over the surface of the rotating star. The flux contributed by each visible source is given by $f = f_0 \cos \theta [1 - B + B \cos \theta]$, where θ is the angle between the line-of-sight and the normal to the stellar surface, f_0 is the flux when viewed normally, and B is the limb darkening parameter. The first $\cos \theta$ factor accounts for foreshortening, while the term in brackets approximates limb darkening for normal stellar atmospheres. The synthetic light curves shown in Figs 3*a* and 4*a* are for a ring of 100 fully darkened ($B = 1$) sources equally spaced around the equator of a spherical star.

The fluxes f_0 are constrained to be positive but otherwise are free to vary in fitting the observed light curve. Equatorial brightness distributions corresponding to the light curve fits in Figs 3*a* and 4*a* are given in Figs 3*b* and 4*b*. In the effort to produce a sharp peak corresponding to the superhump, the stellar brightness distribution breaks up into five precisely arranged bright regions. The structure in the synthetic light curves around $\phi_{\text{sh}} = 0.5$ is also an artefact of this failed attempt to produce a sharp peak. Calculations with sources covering the full stellar surface, with limb darkening parameters in the range $0 < B < 1$, using the correct Roche geometry, and including the eclipse of the red dwarf by the accretion disk, give similar results.

All of the starspot models considered here fail to produce light curves as sharply peaked as the observed superhumps. The starspot models fail because the light curve for a point source on the stellar equator, shown in Fig. 3*a* for undarkened ($B = 0$) and fully darkened ($B = 1$) cases, is already broader than the observed superhump. Any extended brightness distribution must produce an even broader peak. A bright spot at high stellar latitude produces a narrower peak by being visible for less than half of the rotation period, but such spots are ruled out by the absence of secondary eclipses.

The starspot model is thus incompatible with the observed superhump light curves, unless radiation from the starspot is beamed to a considerably greater extent than the radiation from a normal stellar atmosphere. The sharply peaked superhump light curves require the starspot radiation to emerge with an angular full width at half intensity of about 35° , compared with 90° – 120° for limb-darkened stellar atmospheres. As superhumps are seen in both eclipsing and non-eclipsing systems, this beaming must apply only to the direction of stellar longitude. Thus if superhumps arise on the red dwarf, some mechanism must be found to confine the radiation from the bright starspot to a narrow fan beam with its long axis in the direction of stellar latitude.

Light curve synthesis techniques have been used here to clarify the direct constraints that are placed by the light curves of eclipsing SU UMa systems on possible sites for superhumps. These constraints apply without regard to the physical mechanism responsible for superhumps, because they depend only on the well-understood geometry of eclipses in a binary system. Superhump sites at high stellar latitude on the red dwarf are eliminated by the absence of secondary eclipses at $\phi_{\text{orb}} = 0.5$. Sites near the stellar equator are feasible only by invoking a narrow fan beam radiation pattern to explain the sharply pointed shapes of superhumps together with the occurrence of superhumps in both eclipsing and non-eclipsing systems. The shapes of superhumps do not directly constrain disk models, in which the superhumps are produced by time variation rather than by occultation of the superhump source. The absence of deep eclipses of the superhump light in Z Cha, and the shapes of eclipses that occur during the superhump peak independently rule out compact sources on the accretion disk. There is no apparent conflict between the extant eclipse observations and a superhump source that is broadly distributed over the face of

the accretion disk. A more delicate analysis of the eclipse light curves should sharpen the observational constraints on the distribution of superhump light on the face of the accretion disk.

I thank G. Bath, B. Warner and J. Pringle for useful discussions.

Received 5 July; accepted 10 September 1984.

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Differentiation between ^{27}Al nuclei in alums by Fourier transform pure nuclear quadrupole resonance

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Aluminium is the most abundant metal in the Earth's lithosphere and is present in many commercially important materials. Recent studies on its role in zeolites¹ and proposed solar energy storage schemes involving aluminium salts² have led to renewed interest in analytical methods capable of identifying and characterizing specific crystalline sites. In particular, new techniques are necessary which do not require single crystals. One such technique is Fourier transform nuclear quadrupole resonance (NQR) with pulsed field cycling^{3,4}, and we report here on its application to the determination of specific ^{27}Al sites in model aluminium compounds, the alums.

In crystalline compounds, aluminium occurs most often with either four or six nearest neighbours. Tetrahedrally coordinated aluminium is the catalytically active centre in zeolites, where each ^{27}Al atom is believed to be surrounded by four O-Si linkages. The octahedral form, as characteristically found in the alum family, surrounds each ^{27}Al site by six bonds (often to H_2O molecules). Much work has gone into characterizing these sites. In powder samples, the NMR spectra are very broad and the methods used to obtain information have been two-dimensional solid state NMR⁵ and magic angle sample spinning (MASS) NMR⁶. Although the latter technique has proved useful in studies of ^{29}Si in zeolites^{7,8}, its applicability to aluminium is limited because of the small dispersion in ^{27}Al chemical shifts and the line broadening arising from quadrupolar effects, with the result that it generally reveals only the difference between tetrahedral or octahedral sites^{9,10}, except in rare cases of resolution between tetrahedral sites¹¹. As an example, Fig. 1 shows MASS spectra of ^{27}Al in two closely related materials, the ammonium and potassium alums ($\text{NH}_4\text{Al}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ and $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$). This technique reveals no difference between these two samples.

In contrast to high field NMR techniques, magnetic resonance should readily differentiate between signals from similar sites in a zero applied magnetic field; we rely in this instance on the inherent chemical sensitivity of the nuclear quadrupole interaction to resolve structural differences. The quadrupolar interaction arises from the coupling of a non-spherical nuclear charge distribution (characteristic of any nucleus where I , the nuclear angular momentum operator, is less than $\frac{1}{2}$) to molecular and crystalline electric field gradients. In ^{27}Al sites of high symmetry,

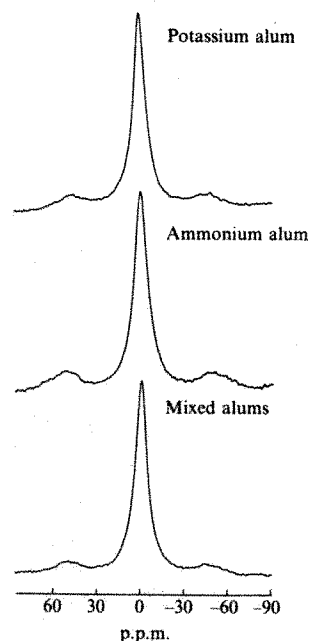


Fig. 1 ^{27}Al magic angle spinning spectra of representative alums. Spectra are observed at 78.2 MHz at a spinning rate of 4 kHz. Chemical shifts are referenced to $\text{Al}(\text{H}_2\text{O})_6^{3+}$. The mixed alum sample is 56 mol% potassium alum, 44 mol% ammonium alum.

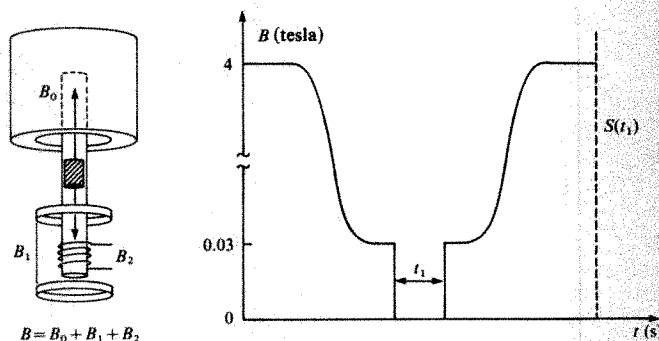


Fig. 2 Schematic diagram showing the effective field at the sample as a function of time. The sample is pneumatically transported from the bore of superconducting magnet B_0 to a point where B_1 accurately compensates for the fringe field. Coil B_2 (0.03 T) is switched off for a time t_1 during which pure quadrupolar evolution occurs. After returning the sample to B_0 , the high field magnetization, $S(t_1)$, is measured. The cycle is repeated for equally spaced points in t_1 until no further change in magnetization is observed. Fourier transformation of the magnetization as a function of t_1 results in the zero field spectrum.

the magnitude of the field gradient term is small. Direct detection of the pure NQR transitions is thus difficult (as the energy splittings are small¹²).

We instead used the chemical sensitivity of pure NQR, in combination with the high signal-to-noise ratio associated with observation of NMR in large applied magnetic fields. This is done by cycling the magnetic field between a large value and zero, and is accomplished in two steps (see Fig. 2): first, the sample is mechanically transported from the bore of a superconducting magnet and then the residual field is pulsed to zero with electronically switched coils. If the second stage occurs quickly relative to the quadrupolar frequencies, the spin system in zero field oscillates at frequencies characteristic of the pure quadrupolar coupling. This oscillation is interrupted at time t_1 by reapplying the field. The sample is then returned to high field where the signal amplitude is monitored. This field cycle is

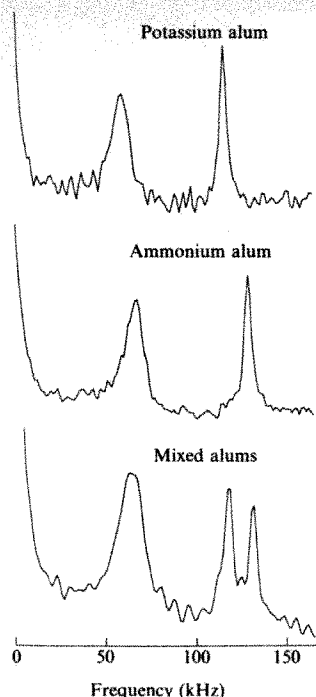


Fig. 3 ^{27}Al Fourier transform pure nuclear quadrupole resonance spectra of representative alums. Each site contributes two lines to the zero field spectrum. η , asymmetry parameter in the quadrupole tensor, was calculated to be 0.17 for potassium alum and ammonium alum. Respective values of 391 and 438 kHz were obtained for the quadrupole coupling constant, $(e^2qQ)/h$, where eQ is the nuclear quadrupole moment, eq is the electric field gradient, and h is Planck's constant. The pair of high frequency lines in the mixed sample (56 mol% potassium alum, 44 mol% ammonium alum) clearly indicate the existence of two distinct ^{27}Al sites.

repeated with different, equally spaced values of t_1 , to give an interferogram which describes the evolution of the spin system in zero field and which, on Fourier transformation, yields the zero field spectrum.

For a spin- $\frac{5}{2}$ nucleus such as ^{27}Al , we expect to observe a pair of lines for each crystalline site. (In aluminium compounds where the crystal symmetry is low, a third line of low intensity may be observed.) Figure 3 illustrates the results for the two alums whose MASS spectra are shown in Fig. 1. Despite nearly identical crystal structures and high field NMR spectra, the zero field nuclear quadrupole experiment clearly differentiates between the two types of aluminium nuclei. We expect that even more sensitive differentiation will be possible between ^{27}Al sites in other systems such as zeolites.

D.B.Z. gratefully acknowledges support by a NSF predoctoral fellowship. This project was supported by the Center for Advanced Materials and the Director, Office of Energy Research, Office of Basic Energy Sciences, Materials Science Division of the US Department of Energy under contract DE-AC03-76SF00098.

Received 1 August; accepted 5 September 1984.

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Direct determination of strontium enrichment on grain boundaries in a garnet lherzolite xenolith by proton microprobe analysis

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The mechanisms by which trace and radiogenic elements are held and transported in the Earth's mantle are of prime importance in studies of basalt petrogenesis and the chemical processing of the mantle. While such mechanisms include the migration of melts and the convective physical admixture of differentiated material¹, the role of fluids as transport agents has attracted much interest, especially, in regions affected by kimberlitic activity^{2,4}, continental basaltic volcanism^{5,6}, and in areas of subduction-related magmatic activity^{7,8}. The observation that many incompatible elements are readily leached from garnet lherzolites by dilute acid treatment³ has led to the assumption that a proportion of these elements is located on grain boundaries, rather than in solution in the various minerals present. This indirect evidence is reinforced by comparison of the compositions of mineral separates with their bulk parental material^{4-8,11}. Although careful electron microprobe analyses have made it possible to detect minor and trace elements within grains¹², the low concentrations involved, together with the high X-ray background of the electron microprobe, have made it impossible to investigate the supposed distribution of incompatible trace elements on grain boundaries. We have measured directly, using a high-resolution proton microprobe^{9,10}, the distribution of Sr and other elements in a garnet lherzolite xenolith. The analyses demonstrate that, in this sample, Sr is preferentially concentrated along grain boundaries.

An inherent limitation in the use of the electron microprobe to analyse very small concentrations of elements lies in the substantial non-characteristic X-ray background caused by electron *bremsstrahlung*. This is very much reduced when a proton beam is used to excite X rays in a sample because the enormously increased mass of the proton minimizes the amount of deceleration near the target. In the present experiments, the specimens were analysed by using a 4.0-MeV proton beam to excite characteristic X rays in the samples. The beam was focused electromagnetically to a spot diameter of 2 μm and was rastered over an area of up to 4 mm $\times$ 4 mm. The samples were prepared as 30- μm thin sections and the X rays generated were detected and analysed using a Link Si(Li) detector system. As the penetration of a proton beam is much greater than that of a beam of electrons in an electron microprobe, the sections were mounted on glass slides using Lakeside 70 thermoplastic resin as a mounting medium. The sections were then removed from the glass slides by gentle heating and remounted over 1-cm holes machined in aluminium target holders to remove any possibility of X-ray generation in the specimen backing. The resulting targets were finally washed carefully in acetone to remove any traces of resin. Samples were irradiated for ~ 10 h using a 50 μm Al filter to cut out the low-energy X-ray background, and maps of the distribution of X rays from major and trace elements in the samples were built up as 256 $\times$ 256 pixel images. As the proton beam penetrated right through the thin samples used, the transmitted beam current was also continuously measured for each pixel so that the effects of any fluctuations in beam current could be corrected. A detailed description of the Oxford proton microprobe system is given elsewhere^{9,10}.

Figure 1 shows elemental maps for the distribution of Ni, Mn, Cr and Sr in a 4 mm $\times$ 4 mm area of a granular garnet

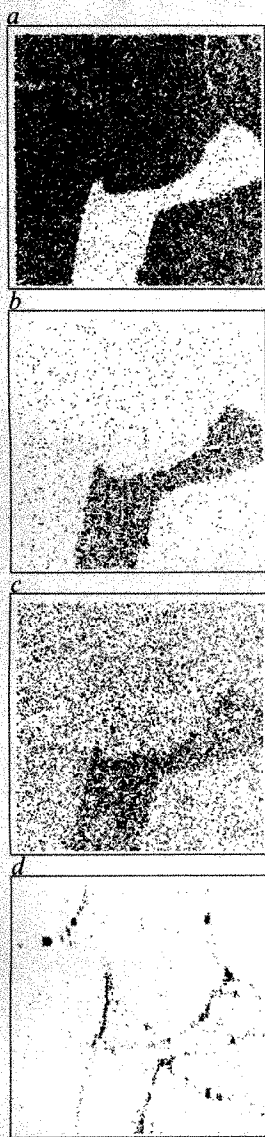


Fig. 1 Elemental X-rays maps of the distribution of Ni (a), Cr (b), Mn (c) and Sr (d) in a 4×4 mm thin section of a coarse garnet lherzolite xenolith obtained using the proton microprobe. Ni, Cr and Mn are present within the mineral grains, but Sr is concentrated on the grain boundaries. Dark areas indicate high concentrations of the elements.

lherzolite xenolith (MK 15) collected from the Bultfontein dumps. The images were displayed in false colour according to the number of X-ray counts per pixel, but are reproduced here as black and white photographs in which the dark areas represent the highest concentrations. The section consists of an angled grain of orthopyroxene (Cr- and slightly Mn-rich and Ni-poor) surrounded by Ni-rich olivine, and there is no obvious zoning in the concentrations of these elements. In marked contrast to the distribution of Cr, Mn and Ni, the map of Sr distribution shows almost no Sr in the interiors of the grains; instead, Sr is concentrated along grain boundaries in the specimen. The Sr was clearly introduced into the rock after its present texture had formed and comparison with the thin section shows that it is located on the serpentinized grain boundaries and cracks in the mineral grains.

To provide an estimate of the concentration of Sr represented in Fig. 1, the data were calibrated by analysing samples of clinopyroxenes which had been analysed previously as mineral separates by isotope dilution¹³. The standards were also prepared as 30- μ m thin sections and data were collected from each standard as the number of Sr-K α counts per nanocoulomb of beam charge deposited on the target. A plot of the count rates against the Sr-contents of the three clinopyroxenes used as standards is given in Fig. 2. The Sr concentrations along the grain boundaries of the garnet lherzolite xenolith, shown in Fig. 1, produced 6–36 counts per nanocoulomb per pixel corresponding therefore to a range of 100–600 p.p.m.

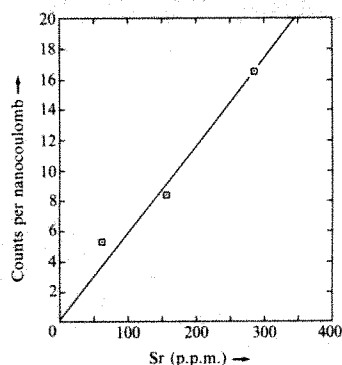


Fig. 2 Calibration of Sr count rates. The number of counts per nanocoulomb of deposited proton charge is plotted against the Sr concentrations of clinopyroxenes which had been analysed for Sr by isotope dilution.

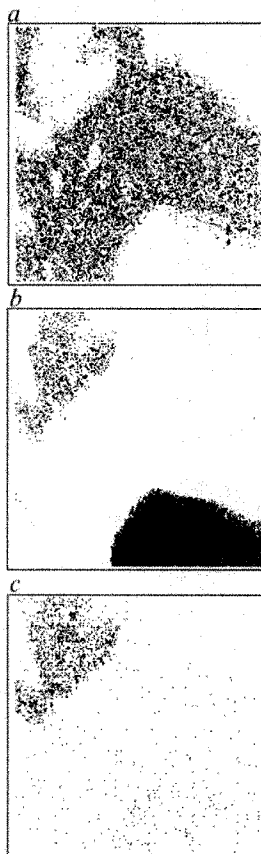


Fig. 3 Elemental X-ray maps of the distribution of Ni (a), Cr (b) and Sr (c) in a thin section of a mosaic porphyroclastic garnet lherzolite xenolith. In this specimen, Sr is distributed like the other trace elements within mineral grains and there is no sign of the process which caused the concentration of Sr on grain boundaries in the specimen in Fig. 1.

Although it is highly unlikely that Sr at these levels could have been introduced into the sample during preparation of a thin section, a second garnet lherzolite sample was selected and prepared in the same way for analysis. This sample (BD 2501) had a mosaic porphyroclastic texture¹⁴, and was known, on the basis of previous studies¹³, to be, of all the xenoliths, one of the least affected by acid leaching. Elemental maps were built up as before and the images for Sr, Ni and Cr in a 4 mm×4 mm section are shown in Fig. 3. The area analysed contains clinopyroxene and garnet grains at the top and bottom of the photographs respectively, separated by a larger Ni-rich olivine grain. The results are in marked contrast to the data shown in Fig. 1 and indicate that, in this sample, there is no detectable concentration of Sr on grain boundaries. Instead, the Sr is present within the mineral grains, the majority being in the clinopyroxene, but with detectable levels in the garnet also. The absence of Sr located along grain boundaries in this section, therefore, makes it unlikely that the data shown in Fig. 1 are an artefact of sample preparation.

These preliminary results provide direct evidence for the location of Sr on serpentinized grain boundaries in certain samples from the upper mantle and strongly suggest the role of an aqueous fluid as the transporting agent. They also indicate the success of the high-resolution proton microprobe as an instrument for determining the concentrations and spatial distribution of trace elements in geological samples. Detailed isotopic work on the timing and nature of the event responsible for the introduction of Sr into the xenolith shown in Fig. 1 will be presented elsewhere (D.G.F. and S. Beswetherick, in preparation).

D.G.F. acknowledges financial support from the NERC and the Anglo-American Corporation. We thank Dr S. Beswetherick for providing the analysed specimens used as calibration points.

Received 18 June; accepted 14 September 1984.

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Upwarp of anomalous asthenosphere beneath the Rio Grande rift

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Continental rifts are possible analogues of mid-ocean ridges, although major plate tectonic features are less clearly observed¹. Current thermal models of mid-ocean ridges²⁻⁴ consist of solid lithospheric plates overlying the hotter, less viscous asthenosphere, with plate thickness increasing away from the ridge axis. The lithospheric lower boundary lies at or near the melting point isotherm, so that at greater depths higher temperatures account for lower viscosity, lower seismic velocities and possibly partial melting. Upwarp of this boundary at the ridge axis concentrates heat there, thus lowering densities by expansion and raising the sea floor to the level of thermal isostatic equilibrium. At slow spreading ridges, a major central graben forms owing to the mechanics of magma injection into the crust⁵. Topography, heat flow, gravity and seismic studies support these models. On the continents, a low-velocity channel has been observed, although it is poorly developed beneath ancient cratons⁶⁻⁹. Plate tectonic models have been applied to continental basins and margins¹⁰⁻¹², but further similarities to the oceanic models remain elusive. Topographic uplift is often ascribed to Airy type isostatic compensation caused by crustal thickening, rather than thermal compensation in the asthenosphere. Here we discuss the Rio Grande rift, in southwestern United States. Teleseismic P-wave residuals show that regional uplift is explained by asthenosphere uplift rather than crustal thickening.

The Rio Grande rift extends for at least 950 km from central Colorado through New Mexico into Mexico. It separates the Great Plains to the east from the Colorado plateau and basin

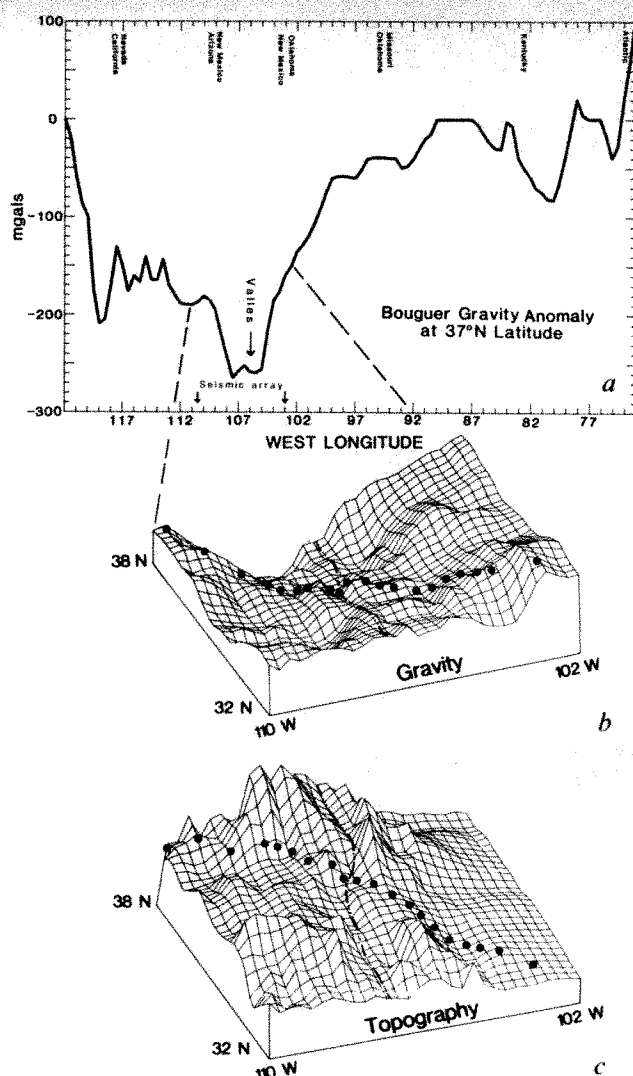


Fig. 1 *a*, East-west Bouguer gravity profile across the United States at latitude 37° N. The Valles caldera and Rio Grande rift lie within the most negative trough. *b*, Three-dimensional perspective view of Bouguer gravity showing locations of teleseismic stations, and Rio Grande River (---). *c*, Three-dimensional perspective of topography showing regional uplift, which is inversely correlated to the gravity of *b*.

and range province to the west and lies just east of the continental divide¹³. Figure 1 shows the transcontinental Bouguer anomaly at 37° N latitude, which crosses the rift along the New Mexico-Colorado border. The rift lies in a region containing the most negative Bouguer anomalies in the continental United States. Figure 1*b* and *c* are three-dimensional perspectives of the regional gravity and topography, which at long wavelengths are inversely correlated. Our experiment investigates whether the basis of the correlation is crustal or asthenospheric.

Rayleigh wave depths to the continental low-velocity zone of the asthenosphere range from about 70 km (refs 8, 9) in the Great Basin of Nevada and western Utah to 83-145 km (ref. 7) in the south-central part of the continent. Although the gravity and topography variations of Fig. 1*b, c* have been attributed^{14,15} to such undulation of lithospheric thickness, the boundary shape has not been determined directly. To probe the Earth to this depth, a high-energy seismic source is required. This is most conveniently supplied by distant earthquakes in the form of teleseismic waves.

The teleseismic P-wave technique¹⁶⁻¹⁹ consists of timing arrivals from distant earthquakes and subtracting from them tabulated²⁰ arrival times for a laterally homogeneous Earth. Any residuals remaining are attributed to lateral heterogeneity

beneath the seismic array, because distant heterogeneities, such as those at the source, will be attenuated by geometric spreading. Incidence and azimuthal angles are calculated from source parameters using a standard Earth model. The pattern of residuals has been observed to shift in response to different incidence and azimuthal angles in the incoming waves. Given enough rays at different angles, the data can be inverted to determine velocity at depths of up to about half the array dimension, with a resolution equal to station spacing near the surface but decreasing with depth.

Figure 2 shows the locations of the 20 recording stations along the 1,000-km line used for the 1982–83 experiment. A north-west–south-east orientation was taken because teleseismic arrivals lie predominantly along this azimuth so that rays from the north-west and south-east intersect in regions lying beneath the array. The array ran from late November 1982 until late January 1983.

Of the 60 earthquakes recorded, 40 have sufficient signal-to-noise ratio to be used in an inversion. Twenty-five of these lie along the array azimuth. The relative P-wave arrival times can be measured to 0.1 s. Residuals are found by subtracting travel times, obtained from the Herrin²⁰ tables, from the arrival times. Relative residuals, formed for each event by subtracting the mean from the raw residuals, are plotted in Fig. 3a. Some of the scatter is due to changes of the residual pattern with the incidence and azimuthal angle.

Additional assumptions must be made if we are to find an unequivocal interpretation for the residual pattern. Therefore, we assume that the regional shifting pattern is mainly due to upwarp of the asthenosphere–lithosphere boundary, that localized heterogeneity gives rise to the small-scale variations, and that the boundary in the vicinity of our array is approximately two-dimensional with a north–south strike. We used synthetic models to test for a way of inverting for such a boundary and decided on two methods: (1) downward projection of the data to give the boundary shape directly²¹ and (2) the two-dimensional^{19,22,23} damped, least-squares block method to give the averaged velocity distribution. Both methods gave similar results (Fig. 3b, c).

The projection method assumes that the interface is not too deep and that velocity contrasts are not too extreme, so that a straight ray and plane wave approximation for the teleseismic waves is adequate. The arrival time residuals will then be proportional to the length of the ray in the anomalous velocity zone, with the proportionality dependent on the velocity contrast. The raw residual pattern gives a distorted picture of the interface—it is projected onto a rotated axis—but as we know the angle of incidence, we can transform the projection to regain the original shape. The transformed patterns are then projected down each ray and the position of maximum overlap determines the true depth. Maximum overlap was determined by minimizing the sum of squares of residuals to a sixth-order polynomial fit by varying both depth and velocity contrast. Figure 3b shows the best-fit model which has a depth range of 70–200 km and a P-wave velocity contrast of –8% relative to a value of 8 km s^{–1} above the boundary. The average misfit of the polynomial to the data was 0.3 s.

The block model involves dividing the region into two-dimensional blocks and determining the velocity perturbations that give the observed delays. After running a series of tests to determine the uniqueness and minimum parameterization of the block model and having found an optimal model, we tested the hypothesis that all the residuals are due to slowness located in the crust or upper lithosphere rather than at depth. The hypothesis was rejected at the 99.9% confidence level: the data require a deep low-velocity zone. Thus, the model we present (Fig. 3c) has interfaces at 40, 70 and 200 km. A block width of 100 km was chosen because our station spacing ranged from 30 to 140 km. The main feature of this model is a spatially coherent 500-km broad, low-velocity zone of –0.35% to –5.1% contrast, in the depth range 70–200 km. Smaller variations in the upper two layers are incoherent. Incoherency was also found

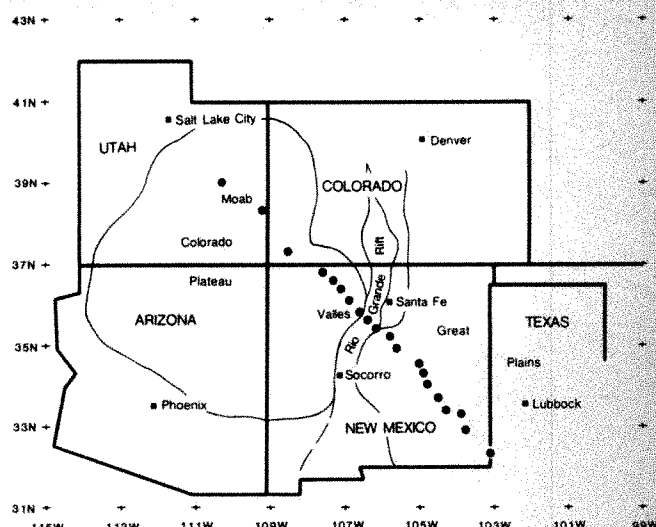


Fig. 2 Station locations. Each consists of a 1-Hz, L4C vertical seismometer and recorder.

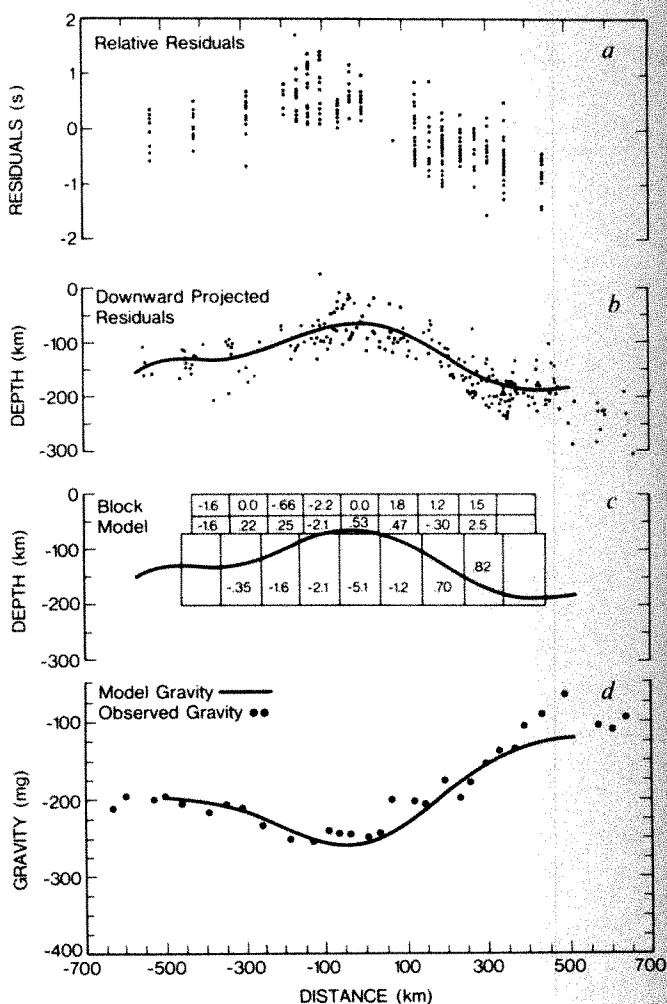


Fig. 3 a, Teleseismic P-wave residuals (de-measured) for 22 events. b, Downward projection of the residual patterns (of a) along ray paths, after rotation to correct for incidence effects. An approximate shape of the inferred lithosphere–asthenosphere boundary is given by the sixth-order polynomial fit. c, Block and polynomial model comparison. Numbers refer to per cent velocity changes compared with –8% contrast found for the polynomial model. Layer interfaces were taken at 40, 70 and 200 km, with unperturbed layer velocities of 6.5, 8.0 and 8.0 km s^{–1}, respectively. d, Gravity anomaly measured along the teleseismic array compared with gravity calculated from the polynomial model of b.

in an additional layer, 130 km thick, when added to the base of the model. The negative anomaly remained unaffected. Standard errors given by the least-squares solution are all $\sim 0.6\%$. The diagonal elements of the resolution matrix ranged from 0.55 in the top layer to 0.86 in the lowest layer. Average misfit for the final model is 0.297 s.

The Bouguer gravity anomaly calculated for both the polynomial model and the block model shows good agreement with the measured data for the area. Figure 3d shows the fit for the gravity values calculated from the polynomial model for which the adjusted density contrast in the low-velocity zone gave a value of -0.05 g cm^{-3} , that is, $\sim 1.6\%$ of 3.2 g cm^{-3} . The ratio of velocity to density contrast, 5.0, is much larger than expected from the Nafe-Drake²⁴ relationship determined from different types of igneous rock, which gives a value of ~ 0.8 . Such an increase in the sensitivity of velocity to change in density is consistent with the presence of a small fraction of partial melt within the low-velocity zone which, while having little effect on density, will have a disproportionately larger effect on shear modulus and hence P-wave velocity. High attenuation supports this view. P-wave amplitudes measured at Albuquerque, which lies on the rift, are among the most attenuated of those measured at standard stations in the United States²⁵. Greater attenuation is seen only at Golden, Colorado, to the north, on the Rocky Mountain front.

The topography also agrees qualitatively with that in isostatic balance with the polynomial model, that is, in thermal isostatic equilibrium. Therefore, the model we assume to explain the teleseismic delays, attenuation²⁵, gravity, uplift^{14,15}, regional heat flow²⁶ and the presence of rifting and volcanism, is based on a hot, low-density asthenospheric body which has replaced the lithosphere by upward movement of the lithosphere-asthenosphere boundary. Such migration of the melting point geotherm might be caused by either lithospheric extension¹⁰ or active convection²⁷ in the upper mantle and requires further quantitative work to elucidate the mechanism. Nonetheless, the boundary has a configuration expected to exist in a region as a prelude to the opening of oceans and continental drift. Although the polynomial fit of Fig. 3b shows some agreement with lithospheric depths determined from Rayleigh waves, other points are still unresolved. Where low P-wave velocities have been associated with low S-wave velocities^{9,28,29} in the low-velocity zone, the per cent reduction in P-velocity is about half that in S-velocity. Our model reduction in P-velocity is -8% , implying an unprecedented regional reduction of S-velocity of -14% . Also, the low-velocity zone has been associated with increased density values^{7,28}, which is opposite to our conclusion. If we compromise between less P-wave velocity perturbation and increased amplitude of the variation of the boundary, the flanks would be placed too deep for them to interface with surface wave models of lithospheric thickness. Therefore, if asthenospheric upwarp is to explain the gravity, topography and teleseismic delays, the properties of the asthenosphere in this region must differ from those encountered elsewhere. However, we might expect this, as it underlies the most negative region of the east-west, continental Bouguer gravity profile (latitude 37° , Fig. 1).

This work was supported by grants from the University of California, Los Angeles, and the Institute of Geophysics and Planetary Physics at Los Alamos National Laboratory.

Received 7 August; accepted 5 September 1984.

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Enhanced preservation of marine-derived organic matter in Cenomanian black shales from the southern Angola Basin

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Black shales possessing high concentrations of organic carbon¹ were deposited in many parts of the proto South Atlantic Ocean during the Cretaceous period². The way such sediments accumulated is not fully understood, but is likely to have occurred through a combination of low oxygen availability and abundant supply of organic matter. Thin, centimetre-thick layers of black shales are commonly interbedded with thicker layers of organic carbon-deficient, green claystones, as found in strata of Aptian to Coniacian age, at Deep Sea Drilling Project (DSDP) Site 530, in the southern Angola Basin³ and elsewhere. These differences in carbon content and colour reflect the conditions of deposition, and possibly variations in the supply of organic matter^{4,5}. We have compared, using organic geochemical methods the compositions of organic matter in three pairs of closely-bedded black and green Cenomanian claystones obtained from Site 530. Kerogen analyses and distributions of biological markers show that the organic matter of the black shales is more marine and better preserved than that of the green claystones.

The results of the analyses are presented in Table 1. X-ray diffraction studies do not show any significant differences between the mineralogical contents of the black and green claystones. Furthermore, the pyrite content of adjacent intervals is similar: its relative abundance seems to depend more on the depositional setting than on the amount of organic matter in the sediments.

Values of total organic carbon, however are significantly higher for the black shales than for their green claystone pairs. Modern deep-sea sediments generally contain $\sim 0.2\%$ organic carbon⁶. Values as high as in these black shales are found in only a few areas of present-day oceans⁷, which are under anoxic or poorly oxygenated bottom-waters. A rare combination of abundant supply and exceptionally good preservation of organic matter is needed for such organic carbon-rich sediments to accumulate.

Visual kerogen analysis of the green and black claystones shows that both contain two populations of vitrinite and amorphous kerogen. Whereas the indigenous vitrinite content indicates that the samples are thermally immature, Fig. 1 shows that the green claystones differ from the black shales in containing a larger amount of recycled vitrinite having a higher thermal

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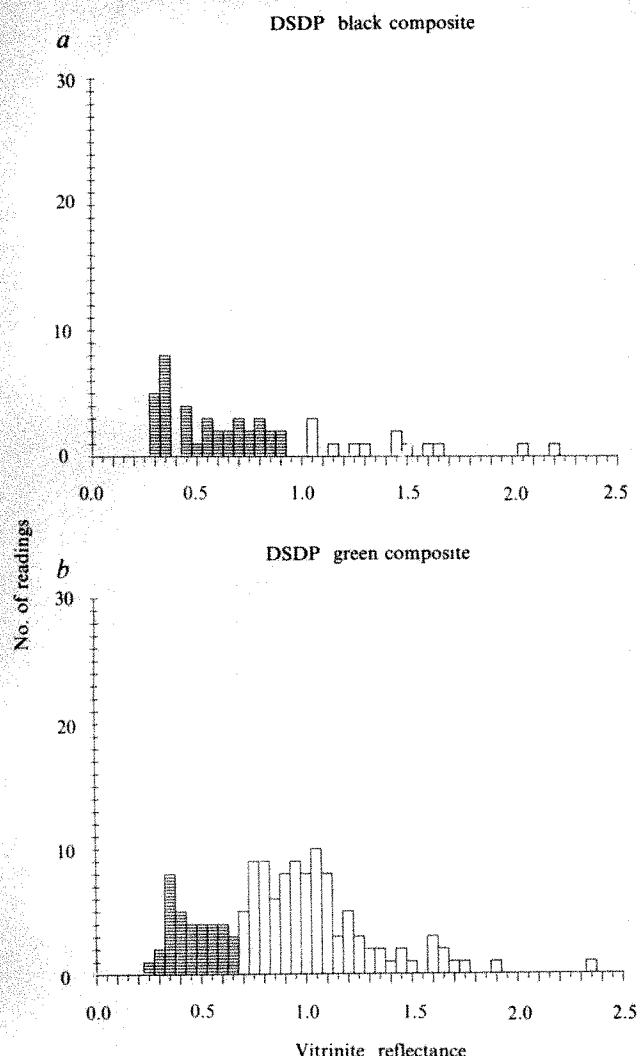


Fig. 1 Vitritine reflectance histograms of composite samples of Cenomanian black shale (*a*, mean = 0.545, median = 0.535, s.d. = 0.195, count = 37) and green claystone (*b*, mean = 0.457, median = 0.450, s.d. = 0.113, count = 35) from DSDP Site 530 in the Angola Basin. Hatched area represents indigenous population.

maturity. Similarly, populations of spores present in the green claystones range from transparent to dark orange, indicating a wide range of thermal maturities, whereas the black claystones contain primarily light-coloured, thermally immature spores. Also the amorphous kerogen of the green claystone is ~85% fluorescent compared with 100% in the black shales. Because thermally immature amorphous kerogen is highly fluorescent, the lower percentages in the green claystones suggest they contain a significant amount of nonfluorescing detrital material. The presence of two populations of both vitritine and amorphous kerogen, indicated as in Fig. 1 by the vitritine reflectance histograms, and by visual inspection, implies there are differences in the organic matter intake and preservation of the two claystones.

The mean value of organic matter C/N atomic ratios is much greater for the black shales (38.1) than for the green claystones (5.7) and is typical of C/N ratios of cellulosic land-plant organic matter⁸. The ^{13}C content of organic matter in black shales from Site 530 is generally less than in green claystones. This difference in isotopic compositions could mean there is a larger component of land-derived organic material in the black than in the green strata. Characterization of organic matter by kerogen analysis, however, shows that the black shales of the Angola Basin contain more amorphous kerogen, indicative of their aquatic origin, than do the green claystones¹⁰⁻¹².

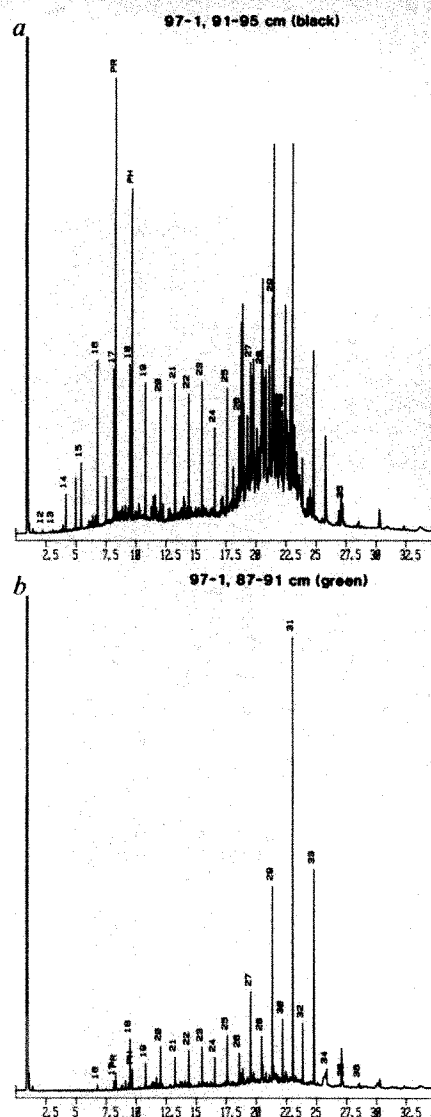


Fig. 2 Gas chromatograms showing extractable aliphatic hydrocarbon distributions of adjacent samples of black (*a*) and green (*b*) claystones from Hole 530A. Pristane and phytane are identified as PR and PH, respectively, and *n*-alkanes by their carbon numbers.

The contradictions in the identification of the source of organic matter, apparent between the C/N ratios and carbon isotope content and the kerogen analyses may arise from selective preservation of organic matter components. Although preservation is generally high in the black shales, nitrogenous materials appear to have been selectively lost, thus enhancing C/N ratios. Similarly, classes of organic compounds relatively rich in ^{13}C , such as amino acids and carbohydrates, may have been selectively degraded, as reported in sediments from Mangrove Lake³, resulting in reduced ^{13}C in the residual organic matter of the black shales. Poorer preservation in the green claystones has led to losses of all but the most resistant forms of organic matter.

Geolipid data agree with this picture of selective preservation. Concentrations of total hydrocarbons, fatty acids, and fatty alcohols are significantly greater in the black shales than in the adjacent claystone (Table 1), yet these geolipids often constitute a smaller fraction of the total organic matter in black shales than in green claystones¹⁴, which is consistent with greater preservation of the more degradable, non-lipid organic matter components in the black shales.

The hydrocarbons in the samples are a combination of compounds either introduced as such to the sediments or generated during diagenesis. The large amounts of aliphatic and aromatic hydrocarbons, in contrast to the smaller amounts of alkanols

Table 1 Comparisons of adjacent Cenomanian black shales with green claystones from DSDP Hole 530A in the Angola Basin

Characteristic of sample	530A-95-5		Sample pairs 530A-97-1		530-97-1	
	0-21 cm	38-50 cm	87-91 cm	91-95 cm	99-105 cm	105-110 cm
Colour	Green	Black	Green	Black	Black	Green
% Calcium carbonate	2	9	5	7	6	6
% Pyrite	4	17	6	6	14	16
% C _{organic}	0.2	12.9	0.6	4.9	10.0	0.8
Atomic C/N	4.5	36.0	6.8	38.1	40.3	5.7
% Amorphous kerogen	60	98	60	98	95	90
% Vitrinite-intertinite	40	2	40	2	5	10
Aliphatic hydrocarbons (p.p.m.)	4	274	20	64	135	18
Aromatic hydrocarbons (p.p.m.)	6	695	20	202	284	126
Alkanoic acids (p.p.m.)	3	7	2	6	11	3
Alkanols (p.p.m.)	0.4	5	2	5	9	3

Samples for geochemical comparison, taken from ~1,020 m sub-bottom depth, were selected onboard ship and immediately frozen to protect their organic matter. Analysis of organic carbon and of solvent-extractable geolipids were done as described elsewhere¹⁴. Kerogen was isolated by demineralization and flotation, and analysed microscopically in both transmitted and reflected light. Bulk mineralogy was determined by X-ray diffraction.

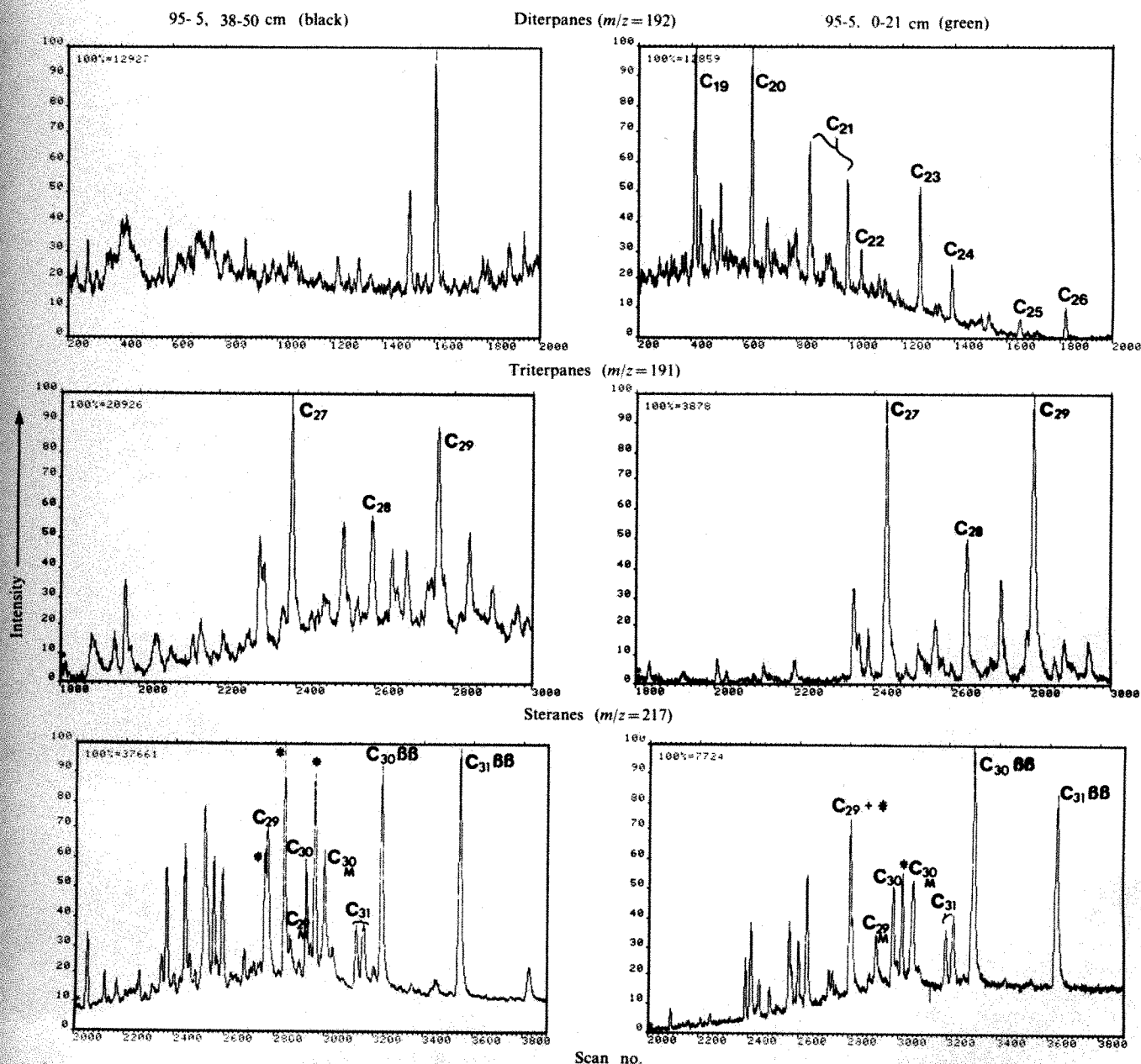


Fig. 3 GC-MS mass fragmentograms of steranes, triterpanes, and diterpanes extracted from black and green claystones from DSDP Hole 530A. The labelled steranes possess unaltered biological 5- α (H), 20- R configurations. The C₂₉ to C₃₁ triterpanes have 17- α (H), 21- β (H) configurations unless labelled as BB. Hopanes are indicated by *, and moretanes (17- β (H), 21- α (H) configurations) by M.

and alkanolic acids in all samples, suggest that a significant amount of these components were generated after deposition and, hence, may reflect diagenetic processes rather than selective preservation as suggested for the more labile compounds. Whereas both types of sample have relatively large amounts of long chain ($>C_{25}$) *n*-alkanes with a pronounced odd-carbon preference, suggesting terrigenous input, Fig. 2 shows that the black claystones also contain significant amounts of shorter chain *n*-alkanes and branched and cyclic hydrocarbons, which are not present in the green claystones^{14,15}. These aliphatic hydrocarbons and the larger quantities of C_{27} relative to C_{29} sterane, which are more evident in the black than in the green claystones (Fig. 3), suggest somewhat more aquatic input to the black claystone. In addition, the presence of diterpanes, predominantly C_{19} and C_{20} , in the green claystone, which are absent from the black claystone (Fig. 3) suggests the input of mostly terrigenous, microbially-altered material to the green claystone^{16,17}. The unidentified peaks in the *m/z* 191 trace of the black claystone probably represent tetracyclic terpanes of unknown origin. Hence, the hydrocarbon analysis agrees with the character of the organic matter intake suggested by the visual kerogen analysis.

The triterpane distributions are quite similar (Fig. 3): both types of sample exhibit abundant 17- β (H), 21- β (H)-hopanes which indicate a low degree of thermal maturity¹⁸. Both the triterpanes present and the occurrence of predominantly biological configurations (that is, 14- α (H), 17- α (H), 20-*R*)¹⁶ of the steranes support the low maturity indicated by vitrinite reflectance and spore colourations, and confirms that the recycled populations do not significantly contribute to the lipid content of these samples.

Comparison of distributions of *n*-alkanoic acids and *n*-alkanols reveals that the black shales contain relatively greater proportions of terrigenous than of marine components of these geolipid classes¹⁴. Because acids and alcohols are more subject to degradation than hydrocarbons, the difference in source character for these geolipid classes again suggests that selective preservation has occurred in the black shales.

The comparisons of organic matter contents confirm that black shales at DSDP Site 530 in the Angola Basin record periods of enhanced preservation of marine, as well as terrigenous, organic material which is superimposed on a low background of residual land-derived material¹⁵. Adjacent green claystones exhibit evidence of burrowing benthic animals and show that bottom waters must have been oxic in Cretaceous times^{3,19}. Because these Cenomanian sediments contain abundant turbidite sequences, the black shales probably represent episodes of downslope redeposition of sediments originally laid down in a midwater anoxic layer which impinged on the African continental slope^{19,20}. Such sediments, rich in organic matter from high marine productivity, must have been rapidly redeposited in the Angola Basin in order to preserve organic substances in an oxic environment. An important implication of this scenario is that deep-ocean black shales can result from local or regional events of short duration which do not involve global oceanic anoxia.

Received 27 February; accepted 13 September 1984.

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A model of estuarine circulation in the Pliocene Mediterranean based on new ostracod evidence

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The recent discovery of the psychrospheric ostracod *Agrenocythere pliocenica* (Seguenza) in Zanclean marls of eastern Crete¹ expands the reach of early Pliocene deep-oceanic circulation far into the eastern Mediterranean. Current models of Pliocene water-mass circulation, though allowing for the Atlantic psychrosphere to flow freely into the western basin, consider the eastern basin as being largely stagnant^{2,3}. I propose here a new model of estuarine circulation affecting both west and east Mediterranean. This circulation was associated with increased atmospheric humidity and driven by upwelling. The model is compatible with organic-rich sedimentation in parts of the eastern Mediterranean and does not imply an excessively deep or wide connection with the Atlantic.

The term 'psychrosphere'⁴ denotes, in an ecological sense, the cold (temperatures $<8-10^{\circ}\text{C}$), dense bottom-waters of the world ocean that are directly derived from the polar regions. The benthic ostracods inhabiting this environment include a group of highly characteristic genera, among them *Agrenocythere*, whose occurrence is virtually restricted to these waters. The depth at which these ostracods occur is generally $>500\text{ m}$ (ref. 2). In lower latitudes, they live at greater depth than in higher latitudes and upwelling regions⁵. *Agrenocythere* is typical of the upper psychrosphere: recent species are most likely to be found at depths of 1,000-1,500 m; the known depth range of the genus is 400-3,850 m (ref. 6).

In the modern Mediterranean, the temperature of deep bottom-water is $\sim 13^{\circ}\text{C}$ ⁷. Hence, recent faunas of this sea do not exhibit any typically psychrospheric ostracods. However, several such forms are recorded from Pliocene and earlier sediments in the Mediterranean region⁸. Figure 1 shows *A. pliocenica* found in Crete¹ which supports an earlier, doubtful identification of a single specimen in the Zanclean of DSDP Site 378 in the south Aegean⁸. All other spots where this species has been found lie much more to the west⁸⁻¹⁰; Sicily and southern peninsular Italy are the easternmost localities known so far. This new find eliminates any doubt that Pliocene psychrospheric circulation did press far into the east Mediterranean.

A. pliocenica first appeared in the Mediterranean in the *Globorotalia margaritae* zone (see Fig. 2 for the epochs, stages and biozones mentioned in this paper). In Italy, this species ranges up to the early Pleistocene¹. In Crete, we have found it

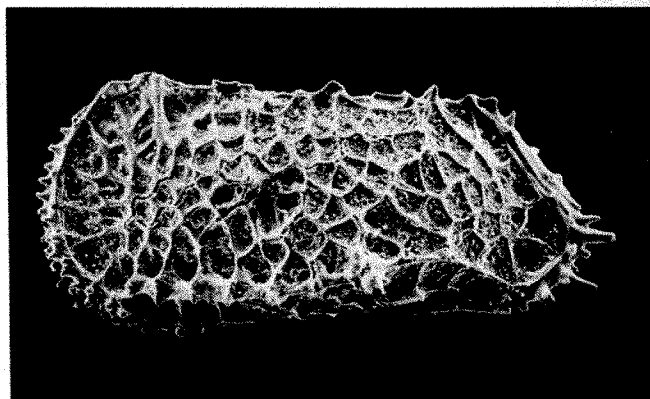


Fig. 1 *A. pliocenica* (Seguenza) from Zanclean marl (*G. margaritae* zone), Xerokambos, southeastern Crete, Greece. Left valve of a male. Length of specimen 1.4 mm.

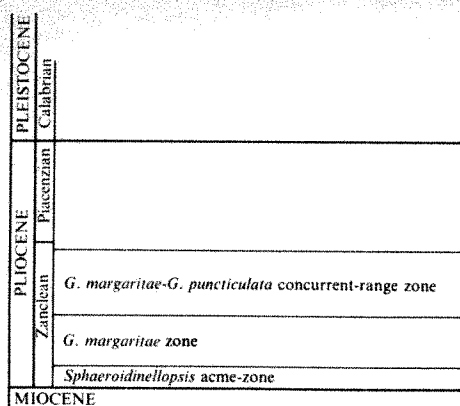


Fig. 2 Shows the relation of epochs, stages and biozones mentioned in the text. The Zanclean is intended to be synonymous with the Tabianian used by other authors to denote the earliest part of a bipartite Pliocene.

so far only in samples referable to the *G. margaritae* zone and possibly the *G. margaritae-G. puncticulata* concurrent-range zone. Psychrospheric conditions, although lasting into the Calabrian in Italy, do not seem to have reached the Zanclean-Piacenzian boundary in Crete.

At present, psychrospheric waters cannot enter the Mediterranean because the sill of the Strait of Gibraltar is too shallow (~320 m). To explain the psychrospheric influence in the Pliocene west Mediterranean, it has been suggested that a relatively wide and deep gateway lay between Spain and Morocco⁵. The sill of the connection with the ocean must indeed have been deeper than it is now, although it need not have been much deeper. Assuming dynamic circulation, a depth of 500–1,000 m must be sufficient to allow the uppermost, cold, deep water layers to pass. The width of the connection is difficult to judge, but it seems unnecessary to claim the existence of a true gateway.

The Sicilian Ridge, the shallow sill between Italy and Africa, divides the Mediterranean into a western and an eastern basin. The sill depth is now ~400 m. But the suggestion is that in the early Pliocene the Ridge was a high-standing barrier to deep circulation and thus encouraged stagnancy and the formation of organic-rich deposits in the eastern basin^{3,11}. This is inconsistent with the present evidence for psychrospheric circulation. The early Pliocene palaeodepth of the sill must at least have been equivalent to that of the strait into the ocean.

Participation of the psychrosphere in the circulation of the Pliocene Mediterranean can be inferred from the mere presence of benthic fauna. Otherwise, the deep water-masses would have stagnated and become uninhabitable through oxygen depletion. The circulation of the Mediterranean is now anti-estuarine (Fig. 3a). The high rate of evaporation, which exceeds the sum of river discharge and precipitation, leads to the formation of dense-surface waters that sink periodically to the bottom (downwelling). The densified Mediterranean water flows out into the Atlantic along the floor of the Strait of Gibraltar. Nearer the surface there is an Atlantic countercurrent which compensates for evaporation⁷. Assuming there is a sufficiently deep connection with the ocean, an estuarine pattern with deep water coming in and surface water going out is the simplest model to include the Atlantic psychrosphere in the Mediterranean circulation (Fig. 3b): upwelling can provide the drive for such a system in much the same way as downwelling operates the modern circulation.

The precondition for estuarine circulation is that the surface waters stay less dense than the deeper waters. This requires a stable pycnocline in the water-column in the form of either a halocline, thermocline, or thermohalocline. Evaporation tends to oppose this by making the surface water both saltier and cooler. An estuarine model therefore requires relatively high atmos-

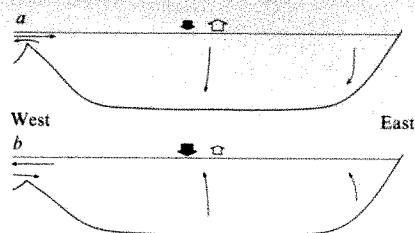


Fig. 3 Mediterranean watermass circulation: paired vertical arrows show schematically the relation of evaporation (open arrows) and fresh-water input by precipitation and river discharge (solid arrows). a, Modern-type anti-estuarine system: surface waters become locally dense and well down, eventually leaving the Mediterranean along the floor of the Strait of Gibraltar; surface currents bring in water from the Atlantic. b, Estuarine system as proposed for the Pliocene and earliest Pleistocene of southern Italy and part of the early Pliocene of Crete: Atlantic upper psychrospheric waters enter the Mediterranean under the influence of a pressure gradient; bottom-waters well up locally and mix with less dense water near the surface; surface currents are directed towards the Atlantic.

pheric humidity to suppress evaporation and promote precipitation. More precipitation leads to increased runoff which, in its turn, could contribute to a primary halocline in parts of the Mediterranean.

The presence of less dense waters in the Mediterranean water-column sets up a pressure gradient with the ocean. If the connection is deep enough, Atlantic psychrospheric waters will enter along this gradient. For these waters to participate in the Mediterranean circulation, upwelling is necessary: wind-driven upwelling most likely occurred in areas off the north coast under the influence of prevailing north winds. Such winds would have tended to move the upper water-layers to the west by Ekman transport. Thermohaline, density-driven upwelling, if at all significant, was probably limited to the easternmost parts where the ocean-derived deep waters had had maximum opportunity to absorb heat.

Estuarine Mediterranean circulation associated with a very warm and humid climate was suggested earlier for the *Sphaeroidinellops* acme-zone¹². Faunas of this zone show an unusual abundance of the planktonic foraminifer *Sphaeroidinellops*³. Similar non-spinose foraminifera with thick tests are known today to prefer deeper water¹³. The abundance of such forms in the earliest Pliocene supports the existence of a halocline with the upper layers too low in salt to be inhabited by marine plankton (S. R. Troelstra, personal communication).

From the occurrence of Taxodiaceae pollen it was recently concluded that humid climatic conditions and rainy summers prevailed in the early Pliocene of the northwest Mediterranean area^{14,15}. An important change, heralding an evolution towards modern seasonal fluctuations (dry summers and wet winters) occurred at the Zanclean-Piacenzian boundary (~3.2 Myr)¹⁵: summer aridity, inferred from the extinction of Taxodiaceae, stabilized diachronously over the Mediterranean region; stability was reached in the Piacenzian in southern France and in the Calabrian in Italy¹⁴. The 3.2 Myr climatic change is also reflected in the oxygen isotope curve of DSDP Site 132 in the Tyrrhenian where it was tentatively associated with increased evaporation relative to precipitation¹⁶.

Sedimentary manifestations of the proposed upwelling might be found in several organic-rich layers or sapropels that have been reported from the early Pliocene of the east Mediterranean, especially at DSDP Site 378 (ref. 17). These sapropels tend to differ from those of the Messinian, late Pliocene, and Quaternary by being relatively low in organic material and burrowed and by containing benthic fauna. They might reflect boosted primary productivity associated with the upwelling of nutrient-rich water from the bottom, rather than complete anoxia due to basin-wide stagnancy¹¹.

Psychrospheric bottom conditions apparently lasted much

longer in Italy than in Crete. At present we can only speculate as to their cause. Considering the similar diachronous summer-aridity stabilization in Italy and France, differential climatic effects may seem a probable cause but direct palynological evidence for this from the east Mediterranean is still lacking. Alternatively, changes in the sea-bottom topography, for instance a rising of parts of the Sicilian Ridge, may have obstructed some deep circulation routes while leaving others unimpaired.

The model outlined here needs further critical testing. Topics to be investigated especially include the climatic history of the east Mediterranean and the relation of Mediterranean organic-rich sediments to upwelling.

I thank Dr Richard H. Benson and Mr Jeroen M. Peters for critical reading of the manuscript and comment. The scanning electron micrograph was made in cooperation with 'Werkgroep SEM', University of Amsterdam.

Received 8 June; accepted 13 September 1984.

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Potassium-selective single channels in guard cell protoplasts of *Vicia faba*

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In plants, stomata control the gaseous exchange between the intercellular spaces of the leaf and the atmosphere. Fluxes of ions, in particular potassium fluxes, across the membranes of the guard cells produce changes in turgor of the guard cells which in turn result in the opening and closing of the stomatal pore^{1,2}. The molecular mechanisms involved in the uptake or release of ions in guard cells are poorly understood². Cell-free membrane patches of the plasmalemma, isolated following patch-clamp techniques³⁻⁵, allow, for the first time in higher plant cells, the separation of the electrical properties of the plasmalemma from those of the tonoplast. We have applied these techniques to study the properties of single-ion channels in the plasmalemma of guard cell protoplasts of *Vicia faba* (broad bean). Predominantly a cation-selective channel was observed, which showed a high selectivity for K⁺, with a permeability ratio P_{K^+}/P_{Na^+} of 11:1 and a single-channel conductance of 37 pS ($=37 \times 10^{-12} \Omega^{-1}$) in symmetrical 225 mM KCl solutions. We estimate that this K⁺ channel contributes significantly to the uptake and release of K⁺ by guard cells during stomatal movement.

Guard cell protoplasts were isolated from the lower epidermis of 3-week-old bean leaves (*Vicia faba* Weisskeimige Hang-down). Epidermal strips were exposed for 60-90 min to 2% cellulase (Rohment CT; Röhm), 0.5% pectinase (Rohment P; Röhm), 0.4 M mannitol and 1 mM CaCl₂. Purified guard cell protoplasts were obtained after 2 h, including the time required

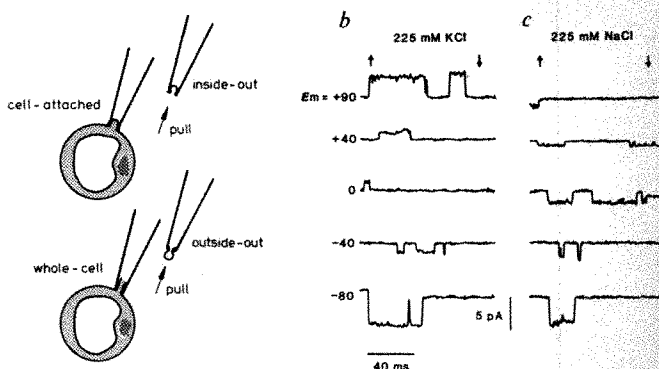


Fig. 1 *a*, Schematic diagram of all four patch-clamp recording configurations³, applied to guard cell protoplasts. Upper panel: high-resistance seals between the pipette and the membrane are established when the fire-polished electrode tip is pressed against the surface of the membrane and suction is applied to the inside of the pipette (cell-attached recording mode). The mechanical stability of the seals allows small membrane fragments to be torn off the cell by drawing the pipette away from the cell. This procedure leaves an intact patch of membrane spanning the pipette tip, resulting in an inside-out patch. The cytoplasmic face of the plasmalemma is exposed to the bath solution. Lower panel: an outside-out patch is obtained when the membrane patch is disrupted by a pulse of suction before pulling off the pipette. The correct sidedness of these patch configurations has been confirmed in numerous experiments on animal cells with channels of known asymmetry³. *b*, *c*, Recordings of K⁺-selective channel currents in an inside-out membrane patch. Discrete open-closed state transitions of single-channel currents can be seen. The membrane was held at $E_m = +40$ mV and stepped by voltage pulses to potentials ranging from +90 mV to -80 mV. The voltage pulse duration was 100 ms. In all records the pulse starts at the upward-pointing arrow and stops at the downward-pointing arrow. Membrane potentials are specified as the potential on the cytoplasmic side of the membrane relative to that on the extracellular side of the membrane during the voltage pulse. Leakage and capacitive currents were subtracted at each potential, using records in which no channel activity was visible. *b*, In symmetrical high K⁺ solutions (225 mM KCl) the single-channel currents are outward at +40 mV and +90 mV (upward deflections of trace). Currents are inward at -40 mV and -80 mV. Note the increase in noise when the channels are open¹⁹. The reversal potential of channel currents is close to 0 mV. Note that on the 0 mV and -80 mV traces, a channel opened before the voltage pulse was turned on and the membrane potential was still +40 mV. When the pulse was turned on, the single-channel current vanished in the 0 mV trace. *c*, After K⁺ was replaced by Na⁺ in the bathing medium (facing the cytoplasmic face of the membrane), the reversal potential shifted to almost +80 mV. A channel was open when the pulse was turned on in the +90 mV trace. All currents are inward from +40 to -80 mV. These data indicate a high selectivity of the channel for K⁺ over Na⁺. Solutions: extracellular (pipette; in mM): 225 KCl, 2 MgCl₂, 1 CaCl₂, 3 KOH, 10 HEPES, 80 D-mannitol, pH 7.0; intracellular (bath): in *b*, same as pipette; in *c*, 225 NaCl, 2 MgCl₂, 1 CaCl₂, 3 NaOH, 10 HEPES, 80 D-mannitol, pH 7.0. All solutions were adjusted to a final osmolarity of $\pi = 500$ mmol kg⁻¹, by adding D-mannitol. Osmolarities were measured with a vapour pressure osmometer (Wescor 5100 C). Temperature 22-25 °C. Control of membrane potential and the measurement of currents were performed with an EPC-7 patch-clamp (List Electronic) and low-pass-filtered at 1 kHz with an 8-pole Bessel characteristic. Data were sampled and stored on a PDP-11/23 minicomputer operating on-line.

for incubation, filtration through a 20 μ m nylon net and two centrifugation steps (60g and 45g). They were suspended in 0.5 M mannitol, 1 mM CaCl₂, stored on ice and used for experiments within 6 h after isolation.

High-resistance seals (>10 G Ω) between the fire-polished tip of the recording pipette and the plasmalemma were established, allowing high-resolution recordings of single-channel currents³. The best results for seal formation were obtained within the first 2 h after protoplast isolation and using small pipettes (tip resistance >3 M Ω)⁶. Techniques well established in animal

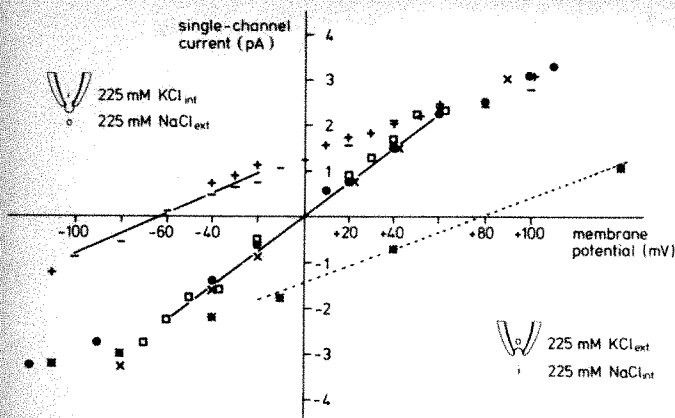


Fig. 2 Amplitude of single-channel currents as a function of membrane potential in three cell-free membrane patches (two outside-out patches, one inside-out patch). The three curves were determined by least-squares regression analysis of data in the given intervals. Outward currents have a positive sign. Middle curve: ●, □ × (× is inside-out patch), data from patches in symmetrical 225 mM KCl solutions. The I - V relationship is approximately ohmic between -60 mV and $+60$ mV. The slope reveals a conductance of 37 ± 4 pS. Left curve and top left insert: The insert shows an outside-out patch spanning the pipette tip. +, - Data from the same two outside-out patches after the bath solution was changed to 225 mM NaCl as in Fig. 1c. The reversal potential of single-channel currents shifted to -61 mV (see text). The slope conductance of single-channel currents was reduced to 22 ± 2 pS (-100 mV to -20 mV). Right curve and bottom right insert: *, Data from inside-out membrane patch after the bath solution (facing the cytoplasmic membrane side) was changed to 225 mM NaCl. The reversal potential shifted to $\sim +78$ mV, corresponding to a permeability ratio P_K/P_{Na} of 21:1. The single-channel conductance was fitted to 19 pS between -10 mV and $+140$ mV. Temperature 22 – 25°C .

physiology were used to produce cell-free membrane patches with either the cytoplasmic membrane face (inside-out patch) or the external membrane face (outside-out patch) exposed to the bathing medium^{3,4} (Fig. 1a). Single-channel current could be studied in these configurations, while defining ionic conditions on both sides of the membrane (Fig. 1b, c).

The most frequent ion channel observed was a K^+ -selective channel (Fig. 1b, c) which appeared in 90% of the patches (patch area 2 – $10 \mu\text{m}^2$). The conductance of this channel depended on the K^+ concentration: in solutions containing 30 mM K^+ on the extracellular and 228 mM K^+ on the cytoplasmic side of the membrane, the single-channel conductance ranged from 20 to 27 pS. The direction of single-channel currents reversed at membrane potentials of $E_m = -50$ mV (reversal potential), corresponding to the Nernst potential for K^+ (not illustrated). In symmetrical high K^+ solutions (both pipette and bath containing 225 mM KCl), the single-channel conductance was 37 ± 4 pS ($\pm$ s.d., $n = 3$) (Fig. 2, middle curve).

The permeability ratio of K^+ to Na^+ was determined by measuring the bi-ionic potential^{7,8}. K^+ in the bathing medium was replaced with Na^+ by bath perfusion (Fig. 1b, 225 mM NaCl); this shifted the reversal potential of single-channel currents from 0 mV to approximately -61 mV in outside-out patches. The single-channel current-voltage (I - V) relationship was approximately linear between -100 and -20 mV with a conductance reduced to 22 pS (Fig. 2, left curve). These data indicate a permeability ratio for K^+ to Na^+ (P_K/P_{Na}) of 11:1, after correcting for ionic activities⁹. When K^+ was replaced by Na^+ on the cytoplasmic side of the membrane in an inside-out patch, the reversal potential of single-channel currents shifted to $\sim +78$ mV, indicating an even higher selectivity for K^+ over Na^+ (Fig. 2, right curve).

The kinetics of opening and closing of the K^+ -selective channel were analysed in a patch in which apparently only one

channel was present¹⁰. The mean lifetime of an open channel at $E_m = -60$ mV was 7 ms ($n = 76$) and 10 ms at $E_m = +60$ mV ($n = 60$). The mean closed time of the channel was ~ 13 ms at both -60 mV and $+60$ mV ($n = 103$ and 72 , respectively). These results indicate that the gating mechanism, responsible for the opening and closing of the K^+ -selective channel, is not significantly voltage-dependent and that the channel is open approximately one-third of the time.

The appearance of channels is not an artefact of patch isolation, as the same K^+ -selective channel was observed in the plasmalemma of intact guard cell protoplasts (cell-attached recording mode)³ (Fig. 1a), as judged by the slopes and reversal potentials of the I - V relationships.

K^+ -salts are the predominant osmotically active compounds responsible for changes in the stomatal aperture in over 50 species^{1,2}. Several authors have described a selectivity for K^+ over Na^+ in conjunction with stomatal movement^{11–13}. Guard cells of *V. faba* accumulate between 100 and 300 mM K^+ when the stomata change from the closed to the open state². Furthermore, the relationship between the resting potential of guard cells and external K^+ concentration has been shown to be approximately nernstian^{14,15}. The mean estimated flux of K^+ into guard cells of *V. faba*, determined by several authors², is 10 pmol s^{-1} per cm^2 of plasmalemma surface. These K^+ fluxes are probably associated with membrane potential shifts of up to 60 mV, caused by effectors on stomatal aperture, such as light, CO_2 and abscisic acid^{16–18}. If the membrane potential is displaced 30 mV from the K^+ equilibrium potential, we predict an initial flux through the K^+ -selective channels in a single guard cell of $20 \text{ pmol s}^{-1} \text{ cm}^{-2}$, corresponding to a change in the cell's K^+ concentration of $60 \mu\text{M s}^{-1}$. In this calculation we assumed a channel density of one channel per $15 \mu\text{m}^2$, a guard cell volume of 5 pl , a single-channel conductance of 30 pS, and that the channel is open one-third of the time. This estimate demonstrates that a significant part of K^+ uptake and release by guard cells can be accounted for by passive transport through the K^+ -selective channel studied here.

In addition to the predominant K^+ channel described, several other types of ion channels were seen but have not yet been characterized properly; patch-clamp studies, as used here, may elucidate their physiological function.

We thank Dr E. Neher, Professor K. Raschke, Dr F. J. Sigworth and Professor D. Gradmann for discussions and comments on the manuscript; and U. Brill, W. Lahr, U. Lohmann, M. Odenthal, W. Pagel, A. Sauer, H. Terasa and F. Tiefenau for peeling bean leaves for protoplast preparation. J.M.F. is an Alexander von Humboldt fellow and J.I.S. was supported by the Deutsche Forschungsgemeinschaft.

Received 23 July; accepted 28 September 1984.

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Benzodiazepines antagonize cholecystokinin-induced activation of rat hippocampal neurones

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Cholecystokinin (CCK) is a neuropeptide present in the mammalian central nervous system (CNS)¹. In all species studied so far, the highest concentrations of this neuropeptide have been found in the cerebral cortex, the amygdala and the hippocampus²⁻⁵. Five molecular forms of CCK having 39, 33, 13, 8 and 4 amino acid residues have been identified in the CNS, the sulphated octapeptide (CCK₈) being the most abundant form detected^{2,6-8}. Specific CCK binding sites have been demonstrated in the rat, guinea pig and human brain^{9,10}. CCK₈, applied by microiontophoresis to deep cortical neurones and hippocampal pyramidal neurones, has a powerful excitatory effect, whereas the non-sulphated CCK octapeptide has no such effect on these neurones¹¹⁻¹⁶. Low doses of benzodiazepines depress the spontaneous activity of hippocampal pyramidal neurones¹⁷⁻²⁰. We report here that benzodiazepines at very low doses antagonize selectively the CCK₈-induced activation of rat hippocampal pyramidal neurones. This antagonistic action might be involved in the anxiolytic effect of these drugs.

Male Sprague-Dawley rats (200-300 g) were anaesthetized with urethane (1.25 g per kg intraperitoneal) and mounted in a stereotaxic apparatus. Five-barrelled micropipettes were prepared in the conventional manner²¹. The central barrel, filled with 2 M NaCl saturated with Fast Green FCF, was used for extracellular recording of unitary activity. Recordings were obtained from pyramidal neurones of the CA1 and CA3 regions of the dorsal hippocampus. The signal, amplified and displayed in the usual manner, was fed into a differential amplitude

discriminator, generating square pulses from which integrated firing rate histograms were obtained. The side barrels were used for microiontophoresis of the following substances: CCK₈ (10 μ M in 0.2 M NaCl, pH 5); acetylcholine chloride (20 mM, pH 4); flurazepam-2HCl (20 mM, pH 4); chlordiazepoxide-HCl (10 mM in 20 mM NaCl, pH 3); Met-enkephalin (0.5 mM in 0.2 M NaCl and 0.01% bovine serum albumin, pH 4.6); L-glutamate (50 mM in 50 mM NaCl, pH 8.0); L-aspartate (50 mM in 50 mM NaCl, pH 8.0). Three side barrels were used for ejection of the test substances and the fourth one, filled with 2 M NaCl, was used for current balancing. Recording sites were marked by passing a -27 μ A current through the central barrel for 20 min, to make a Fast Green deposit, the location of which was verified histologically.

Intravenous (i.v.) administration of flurazepam, lorazepam or diazepam during the activation of 18 hippocampal pyramidal neurones by microiontophoretically applied CCK₈ reversed this activation, whereas the excitatory effects of applications of acetylcholine (ACh) (14 neurones) or Met-enkephalin (7 neurones) were not affected (Fig. 1a). The effect of the three benzodiazepines was the same in the CA1 and CA3 regions. From the dose-response curves of the effect of i.v. lorazepam and diazepam on CCK₈-induced activation (Fig. 2), we calculated the 50% effective dose (ED₅₀) to be 32.6 ± 2.8 (mean $\pm$ s.e.m.) and 106.5 ± 18.6 μ g per kg for lorazepam and diazepam, respectively.

To verify further the selectivity of benzodiazepines for CCK₈ and to determine whether they were reversing CCK₈-induced activation by a direct action on the pyramidal neurone recorded, flurazepam or chlordiazepoxide, two water-soluble benzodiazepines, were applied microiontophoretically during the activation of CA1 or CA3 hippocampal neurones by applications of CCK₈ (40 neurones), Met-enkephalin (14 neurones), glutamate (8 neurones) and aspartate (8 neurones). Microiontophoretic applications of either benzodiazepine reversed the CCK₈-induced activation of pyramidal neurones but not that produced by ACh, Met-enkephalin, glutamate or aspartate (Fig. 1b-d).

As a preliminary investigation of the specificity of this antagonism for benzodiazepines, haloperidol (5 mg per kg), an

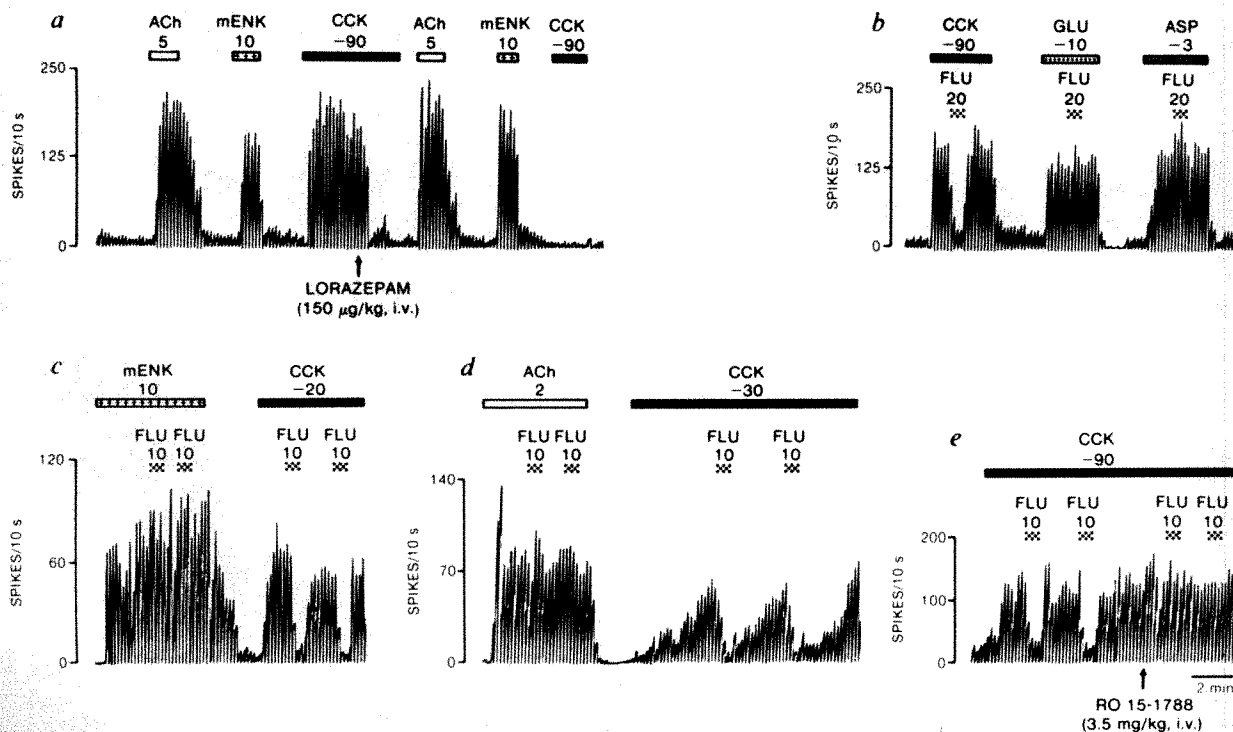


Fig. 1 Histograms of integrated firing rate for pyramidal neurones of the dorsal hippocampus CA3 region. Bars indicate duration of microiontophoretic applications, for which the current is given in nA. The time scale in *e* applies to all the traces. CCK, cholecystokinin-8; ACh, acetylcholine; mENK, Met-enkephalin; FLU, flurazepam; GLU, glutamate; ASP, aspartate.

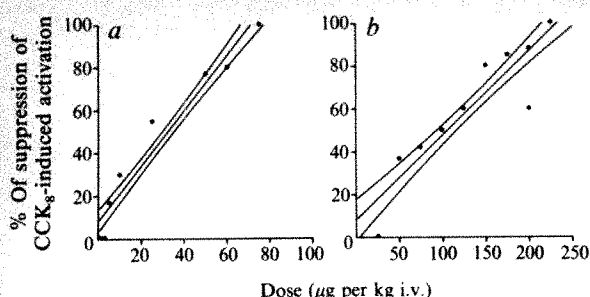


Fig. 2 Dose-response curve ($\pm$ s.e.m.) of the degree of suppression by i.v. lorazepam (a) and diazepam (b) of the activation of hippocampal pyramidal neurones induced by microiontophoretic application of CCK₈. $r = 0.94$, $P < 0.001$ for lorazepam; $r = 0.82$, $P < 0.001$ for diazepam.

antipsychotic drug, phenobarbital (10 mg per kg), an anticonvulsant barbiturate, and meprobamate (20 mg per kg), a non-benzodiazepine anxiolytic drug, were administered i.v. during the activation of CA3 pyramidal neurones by CCK₈. None of the three non-benzodiazepine drugs affected the CCK₈-induced activation in the 10 experiments performed.

To ascertain that benzodiazepines antagonize CCK₈-induced activation via their specific receptors^{22,23}, we used the imidazodiazepine Ro 15-1788, a benzodiazepine antagonist²⁴. Ro 15-1788 (3.5 mg per kg i.v.) completely blocked the effects of microiontophoretic applications of flurazepam and chlordiazepoxide and of the i.v. administration of lorazepam (100 µg per kg) on CCK₈-induced activation in all six rats tested (Fig. 1d). Moreover, in four other experiments, Ro 15-1788 (3.5 mg per kg i.v.) completely reversed the antagonism of the CCK₈-induced activation produced by microiontophoretic application of flurazepam and i.v. lorazepam (not shown).

This is the first electrophysiological demonstration of the antagonism of the action of a neuropeptide on CNS neurones by benzodiazepines. The present data do not allow any conclusion regarding the molecular mechanism underlying the antagonism by benzodiazepines of the neuronal activation by CCK₈. The selectivity of the action of benzodiazepines for CCK₈-induced activation is indicated by their inability to affect, when administered either intravenously or microiontophoretically, the excitatory effects of another peptide, Met-enkephalin, of ACh and of two putative neurotransmitter amino acids, glutamate and aspartate. Benzodiazepines have been shown to antagonize the excitatory action of kainate on CA1 hippocampal pyramidal neurones²⁵. However, doses more than 30 times greater than those used here are required to reduce kainate-induced activation²⁵; moreover, the activation of CA3 pyramidal neurones by kainate is not antagonized by benzodiazepines²⁵ whereas the excitatory action of CCK₈ is blocked equally well by benzodiazepines in the CA1 and CA3 regions.

The activity of all four benzodiazepines tested in the present study (flurazepam, diazepam, lorazepam and chlordiazepoxide) suggests that the ability to antagonize CCK₈-induced activation is a property common to all drugs of this class. The inactivity of the three anxiolytic non-benzodiazepine drugs tested (haloperidol, phenobarbital and meprobamate) constitutes a preliminary indication that this property might be specific to benzodiazepines. That the antagonism of CCK₈-induced activation might be a property common to, and specific for, benzodiazepines is fully consistent with the prevention and the reversal by Ro 15-1788, a specific 'neuronal' benzodiazepine antagonist, of the effect of benzodiazepines on CCK₈-induced activation²⁶. As there is strong evidence that the activation of the neuronal type of benzodiazepine receptors mediates the anxiolytic effect of benzodiazepines^{27,28}, this suggests that the antagonism of CCK₈ action by these drugs might be involved in their therapeutic effect. The extreme potency of benzodiazepines in blocking CCK₈-induced activation is consistent with this hypothesis.

It is indeed striking that the ED₅₀ for lorazepam (32 µg per kg) and diazepam (106 µg per kg) found here are very close to the most widely prescribed single doses of 1 mg lorazepam and 5 mg diazepam (equivalent to 15–20 µg per kg and 75–100 µg per kg, respectively, for an adult of average weight) in the treatment of anxiety. Moreover, the ratio of the potencies of lorazepam and diazepam found here is very close to that of their clinical anxiolytic activities. Finally, the high density of CCK₈ in the cerebral cortex and in limbic regions such as the hippocampus and the amygdala^{2–4} makes this neuropeptide a suitable candidate for mediating the selective anxiolytic effects of benzodiazepines. As benzodiazepine receptors are coupled with γ -aminobutyric acid (GABA) receptors²⁹, there is a need to investigate the involvement of GABA in mediating the selective suppression by benzodiazepines of CCK₈-induced activation.

We thank W. Haefely for providing us with Ro 15-1788, G. Dubé for typing, and D. Cyr and G. Filosi for preparing the illustrations. C. de M. is a Scientist of the MRC of Canada and J.B. a Fellow of the Fonds de la Recherche en Santé du Québec.

Received 11 June; accepted 10 September 1984.

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Immunohistochemical localization of endogenous nerve growth factor

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Nerve growth factor (NGF) has been proposed as a trophic molecule essential for the development of sympathetic and primary sensory neurones^{1,2}. In newborn mice and rats, administration of nerve growth factor results in an increase in the number of surviving neurones^{3,4}, whereas administration of antiserum to NGF decreases neuronal survival^{5,6}. Thus it has been proposed that the factor is produced and secreted by the relevant target tissues to provide trophic support for the ingrowing nerves^{1,7}. The site of synthesis of nerve growth factor is still unknown, and it has been emphasized

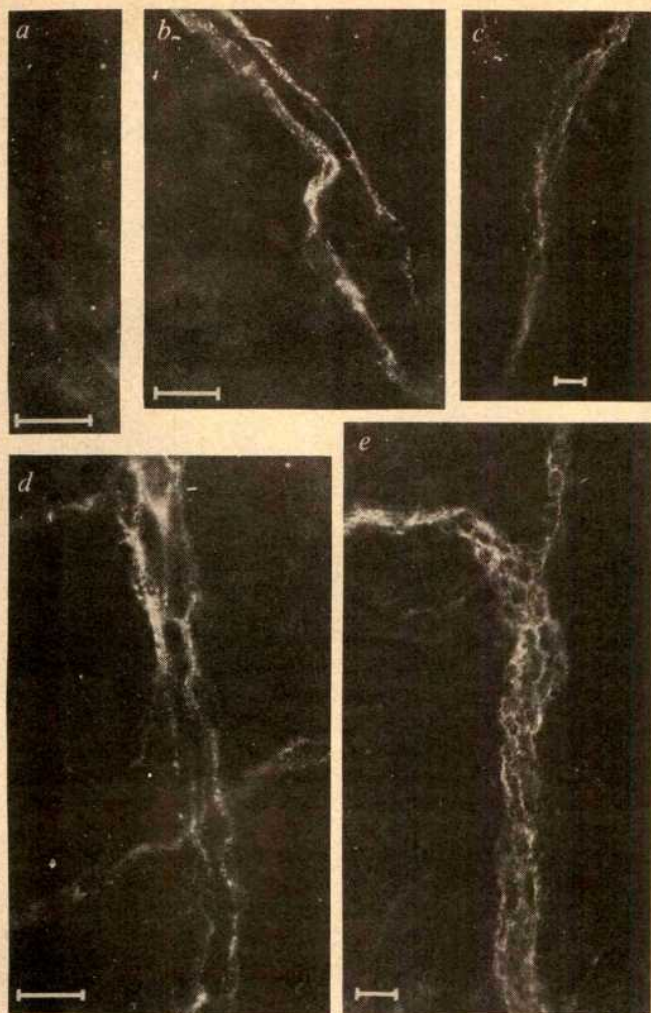


Fig. 1 Anti-NGF immunohistochemistry in the rat iris. *a*, The absence of stain in the non-cultured iris; *b*–*e*, the presence of stain in cells clustered into bundles following 4 or 6 days culture. Irises were cultured for 4 (*b*, *d*) or 6 (*c*, *e*) days in Eagle's basal medium containing 10% fetal calf serum and exposed to a humidified 5% CO₂/95% air atmosphere. After incubation the irides were removed, freeze thawed and stretched on polyornithine-coated glass slides and allowed to dry for at least 30 min at room temperature. Antiserum, prepared against mouse salivary gland NGF (electrophoretically pure), was diluted 1 in 4,000 in phosphate buffered saline (PBS) pH 7.4 containing 1% sheep serum and applied to the stretched tissues. Following 48 h incubation at 4 °C, the antiserum was removed and replaced by several changes of PBS. Fluorescein-labelled anti-rabbit IgG (Wellcome, Sydney) was used at a dilution of 1:100 and applied for 1 h then washed from the tissue before mounting. Scale bar, 10 μ m.

that a precise physiological role for the molecule cannot be ascribed until the cell types that produce it are known^{7–9}. I report here the use of immunohistochemistry to localize endogenous NGF in the rat iris, a tissue in which there is sound biochemical evidence for the production of NGF activity¹⁰. Surprisingly, the results reveal that NGF can be detected readily in Schwann cells, but not in smooth muscle cells of the iris when it is sympathetically denervated or cultured.

Irises from adult albino rats (250–300 g body weight) of both sexes were used. After culture for four or six days in Eagles basal medium supplemented with 10% fetal calf serum, irides were removed, freeze-thawed and stretch-mounted on glass slides. Specific stain was clearly visible in all regions of the iris confined to discrete clusters of cells (Fig. 1). The distribution of these cells was similar to that of nerve fibres and bundles throughout the normal iris. Although many labelled cells were clustered in large bundles, individual fusiform cells were also

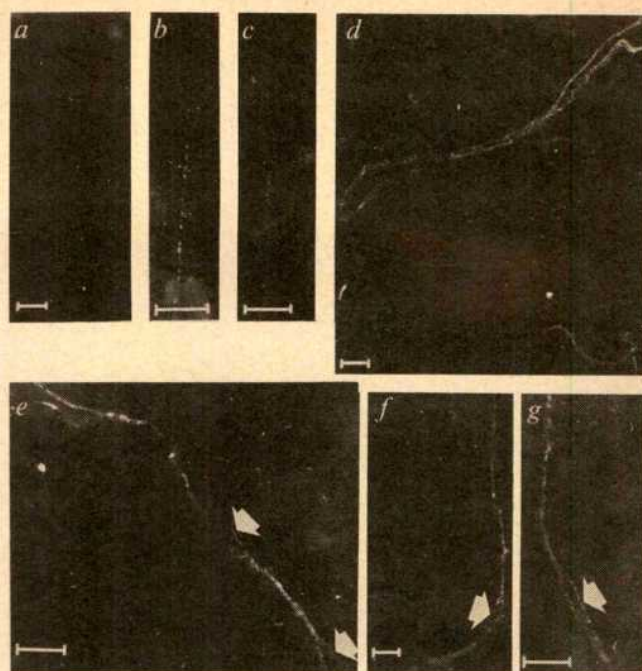


Fig. 2 Immunohistochemistry in the 6-day cultured rat iris. *a*, Absence of stain in tissue treated with pre-immune serum. *b*, *c*, Presence of stain in degenerating nerve fibres treated with dopamine β -hydroxylase antiserum. *d*–*g*, Presence of stain in strings of single cells stained with nerve growth factor antiserum. Arrows, nuclei discernable by the absence of stain. Staining procedure as for Fig. 1. Scale bar, 10 μ m.

present and were joined end-to-end in long strings with nuclei easily discernable by the absence of stain (Fig. 2). The resolution of the light microscope did not allow the exclusion of stain in degenerating nerve fibres. Even after four days of culture there were a few fragmented sympathetic nerve fibres present that could be visualized by immunohistochemistry with antiserum to dopamine β -hydroxylase¹¹ (Fig. 2). At no stage was anti-NGF stain visible in any smooth muscle fibres; irides that were processed without prior incubation gave negative results (Fig. 2). Cultured and non-cultured irides treated with pre-immune serum were also negative (Fig. 1). Identical results were seen in tissues fixed in picric acid solution prior to staining (Fig. 3). The specificity of the nerve growth factor antiserum was tested (Fig. 3); absorption of the antiserum with NGF-coupled beads blocked the immunohistochemical stain whereas absorption with insulin-coupled beads had no effect. Furthermore, the immunohistochemical stain could be demonstrated using dialysed antibody eluted from the NGF-coupled beads with 1 M acetic acid (Fig. 3).

Irises were next examined following removal of the ipsilateral superior cervical ganglion ten days previously. The effectiveness of sympathetic denervation was demonstrated in half of the iris by the absence of fluorescent nerve fibres following treatment with glyoxylic acid to induce catecholamine fluorescence¹². The remaining half of the iris was stained with NGF antiserum. The distribution of stain was similar to that seen after culture, although both the intensity of the stain and the number of positive cells was reduced considerably (Fig. 4). Contralateral irides treated with the antiserum were negative.

The results presented here provide the first histochemical evidence for the cellular localization of endogenous NGF outside the salivary gland. It is important therefore to examine the authenticity of the antisera used. The major antiserum was raised in rabbits against electrophoretically pure mouse salivary gland nerve growth factor prepared by the method of Mobley *et al.*¹³ (Fig. 4). The purified protein was active in the 1–10 ng ml⁻¹ range, tested by supporting dissociated chick sympathetic

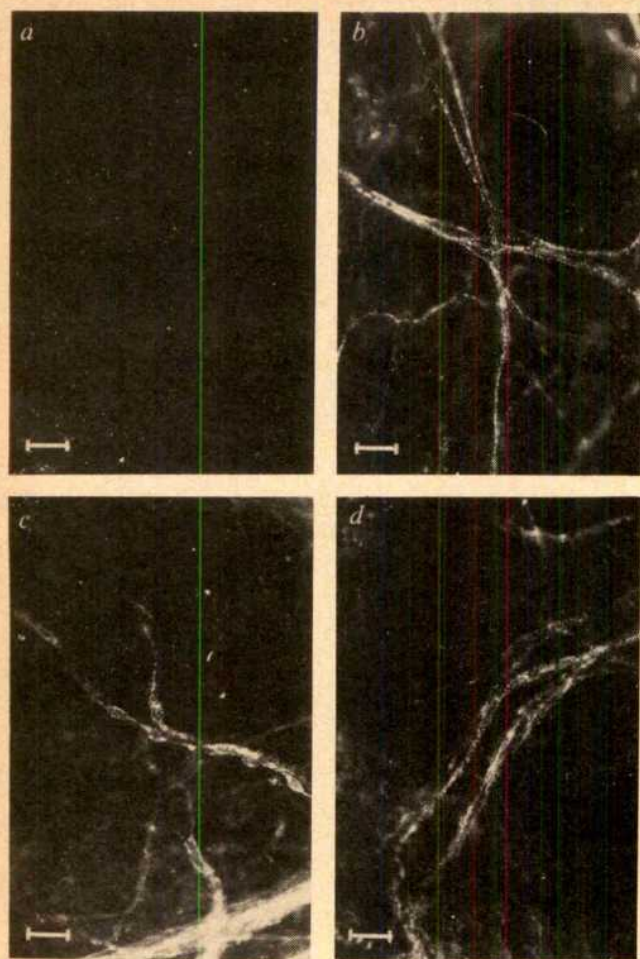


Fig. 3 Immunohistochemistry in the 6-day cultured rat iris. *a*, Absence of stain in an iris treated with antiserum previously absorbed with NGF-Sepharose; *b*, the presence of stain after treatment with whole antiserum; *c*, the presence of stain after treatment with antiserum absorbed with insulin-Sepharose; *d*, the presence of stain after treatment with antibody eluted from the NGF-coupled beads. Irises in *a*, *c* and *d* were fixed after freeze-thaw treatment and prior to staining in picric acid solution for 30 min (150 ml of saturated picric acid solution in 850 ml of PBS, pH 7.4); the iris in *b* was stained without prior fixation as described in Fig. 1.

Methods: Absorption of the antibody with solid phase antigen was performed as follows. Antiserum diluted to 1 in 4,000 was added to 100 μ l Sepharose 4B (Pharmacia) coupled to either NGF or insulin and incubated for 30 min at 37 °C in a shaking water bath. The supernatant was then removed from the beads and used immediately for immunohistochemistry. Purified antibody was eluted from the NGF-Sepharose with 1 M acetic acid and dialysed against PBS before use at a final protein concentration of 2.5 μ g ml⁻¹. Coupling of NGF and insulin to epoxy-activated Sepharose 4B was carried out according to the manufacturer's recommendations using 2 mg of each protein with 0.5 g of lyophilized gel. Scale bar, 10 μ m.

neurons in culture, elevating ornithine decarboxylase activity in newborn rat superior cervical ganglia and in increasing the number of surviving neurons in sympathetic ganglia when administered to developing chick embryos. The resulting antiserum was monospecific against NGF, shown by immunoelectrophoresis against both the purified factor and crude homogenates of male mouse salivary glands; it also stains granular duct cells in this tissue (Fig. 4). This antiserum blocks the survival of sympathetic neurons cultured in the presence of NGF¹⁴. Antiserum and commercial antibodies (Laref, Switzerland), purified by chromatography on an NGF affinity column, gave identical results whereas normal sera from several different rabbits gave negative results. Thus the staining in the figures is

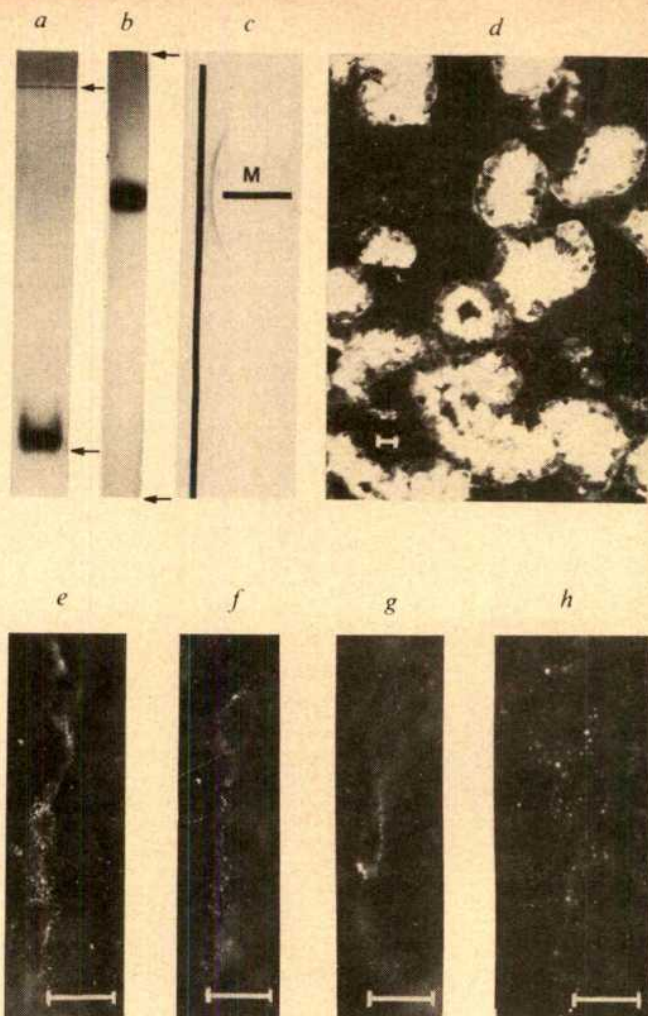


Fig. 4 *a-d*, Evidence for specificity of antiserum to NGF; *e-h*, NGF immunohistochemistry in the sympathetically denervated iris. *a*, Electrophoresis of purified mouse salivary gland nerve growth factor on 12.5% polyacrylamide discontinuous gels in SDS according to the method of Laemmli and Favre²⁰ with a protein load of 30 μ g. *b*, 20 μ g purified NGF on a 30% polyacrylamide gel, pH 10.3, according to the method of Smith, Varon and Shooter²¹. Arrows, position of the origins and dye fronts. *c*, Immunoelectrophoresis against crude mouse salivary gland extract. Electrophoresis was carried out on Corning Universal electrophoretic agarose film (Corning) in 0.065 M barbital buffer, pH 8.6 for 45 min at 200 V with constant cooling. Under these conditions, mouse salivary gland 2.5S NGF does not migrate significantly from the origin. The film was stained after washing with Coomassie blue. *d*, Immunohistochemical localization of NGF in submaxillary gland of male mouse. Anaesthetized male mice were perfused with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, the submaxillary glands removed, cut into small blocks and immersed for 3–4 h in the same fixative. The tissue blocks were then rinsed overnight in 5% sucrose in 0.1 M phosphate buffer pH 7.4 and sectioned at 10 μ m on a cryostat. Sections were stained for 48 h at 4 °C with anti-NGF serum diluted to 1 in 4,000 as described in Fig. 1. The fluorescent stain was most intense in apical regions of granular duct cells and absent in acinar cells. *e-h*, fluorescent cells stained with anti-NGF in irides sympathetically denervated 10 days previously. Scale bar, 10 μ m.

probably a result of the interaction of NGF antibodies with an endogenous NGF-like molecule.

The staining present in denervated or cultured irides is localized to a small proportion of the total cells present that are confined to nerve fibre tracts. I suggest here that these are Schwann cells and possibly associated nerve fibres. In the absence of electron microscope evidence, there are several rea-

sons for this conclusion. First, the stained cells clearly are not smooth muscle fibres as their morphology and distribution are distinct from these cells. Second, their morphology is identical to Schwann cells and they are arranged either in clusters or as elongated single cells in bundles resembling nerve fibre bundles. Third, although fibroblasts are present in nerve bundles, their total number is only about 5% of the number of Schwann cells¹⁵. It is unlikely that fibroblasts would be confined to nerve bundles and yet no isolated stained cells were observed outside the bundles. However, this does not exclude the possibility that some positive cells are fibroblasts. Finally, the production of NGF by purified cultures of Schwann cells^{16,17} and isolated peripheral nerve segments¹⁸ is established clearly.

Although it is clear that the anti-NGF stain is associated with nerve tracts throughout both the radial and sphincter muscles, it was not possible to establish which type of nerves are involved. Specific stain was present in both fine nerve tracts and thick bundles; the density of the fibre tracts stained throughout the iris was only slightly less than the density of sympathetic fibres in a non-cultured iris revealed by glyoxylic acid-induced catecholamine fluorescence. In these cultured irides the nerve axons and terminals detached from their cell bodies are degenerating; immunohistochemical localization with dopamine β -hydroxylase antiserum has confirmed that many of the sympathetic nerve fibres have been lost. However, experiments are currently in progress to double-label tissues with NGF and substance P, dopamine β -hydroxylase or choline acetyltransferase antisera to discover whether sensory, sympathetic or parasympathetic fibres are involved.

The production of NGF by Schwann cells in the denervated iris suggests that damaged nerves receive trophic support in the old fibre tracts during regeneration. It also suggests that after injury the nerve returns to an embryonic-like sensitivity. Certainly, regenerating sympathetic nerves are sensitive to NGF⁴, whereas intact nerves in the mature animal show little response to the factor⁷.

Many target tissues of the sympathetic and sensory nervous systems are known to produce NGF in culture¹⁹. This observation has been used to strengthen the proposal that neuronal death occurs during development as a result of competition between growing axons for NGF secreted from target cells, such as smooth and cardiac muscle fibres. However, extrapolation of the present findings to developing nerves would suggest that the nerves are supported during the period of axonal outgrowth by NGF from Schwann cells and that at the time of innervation, the target cells provide a second trophic molecule that is not NGF.

I thank Miss Fiona Renton for technical assistance and Dr Ian Hendry for supplying a sample of antiserum to nerve growth factor. The work was supported by the NH and MRC of Australia and by equipment grants from the MDA of South Australia and the Flinders Medical Centre Research Foundation.

Received 17 August 1983; accepted 11 September 1984.

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Aetiology of AIDS—antibodies to human T-cell leukaemia virus (type III) in haemophiliacs

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Human T-cell leukaemia virus type III (HTLV-III) is suspected of having a key role in the pathogenesis of acquired immune deficiency syndrome (AIDS)¹⁻⁴. Epidemiological data suggest that AIDS is transmitted by an infectious agent through intimate contact with body secretions, blood or blood products⁵. To maintain haemostasis, many haemophiliac patients depend on commercially prepared clotting concentrates made from large multi-donor plasma pools and are thus at increased risk of developing the disease^{6,7}. We report here that, using indirect membrane immunofluorescence and radioimmunoprecipitation with SDS-polyacrylamide gel electrophoresis, we have detected antibodies to HTLV-III in 30 of 47 (64%) asymptomatic haemophiliacs and in all of three haemophiliacs who either had or soon developed AIDS. Of 34 samples drawn before 1984, 18 (53%) were antibody-positive, whereas of 16 samples drawn during 1984, 15 (94%) were positive ($P \leq 0.002$). These data suggest that exposure to HTLV-III antigens is widespread among asymptomatic haemophiliacs.

We tested samples from 50 haemophiliacs from the New England Area Comprehensive Hemophilia Center in Worcester, Massachusetts; 44 of the patients had haemophilia A and 6 had haemophilia B. All haemophilia A patients had moderate or severe disease and received home infusions of commercial factor VIII concentrate. The samples were collected during 1983-84 from individuals ranging in age from 1 to 69 yr. All were asymptomatic at the time of serum collection, except for a single patient who had clinical AIDS. Two more individuals developed AIDS within 3-6 months (confirmed by the Center for Disease Control).

All sera were initially examined at a 1:4 dilution by indirect membrane immunofluorescence (IMI) on the HTLV-III-infected line, designated H9/HTLV-III, and the uninfected line, designated H9. The method used was that described earlier, except that the cells were inactivated with 2% paraformaldehyde in phosphate-buffered saline as a biohazard precaution before the tests were scored. All 50 samples were subsequently tested using radioimmunoprecipitation with SDS-polyacrylamide gel electrophoresis (RIP-SDS/PAGE). Both uninfected H9 and HTLV-III-infected cells were metabolically labelled for 8 h with ³⁵S-cysteine (100 μ Ci ml⁻¹) according to details described elsewhere^{8,9}.

Of 50 serum samples tested, 33 (66%) reacted with the HTLV-III-infected cells by IMI, but not with the uninfected cells (Table 1, Fig. 1). The same 33 sera showed immunoprecipitation of proteins that migrate in the same position as those regularly detected with reference antiserum to HTLV-III core proteins (provided by Dr R. C. Gallo). Samples from 29 healthy laboratory workers were all negative when tested by both membrane immunofluorescence and immunoprecipitation. The antigens most consistently detected by the 33 positive sera included proteins of molecular weights 24,000 (p24), 41,000 (gp41), 53,000 (p53), 120,000 (gp120) and 160,000 (gp160); these are the proteins that Gallo and colleagues¹⁻⁴ and workers in our laboratory (T. H. Lee and M.E., manuscript in preparation) have found are most frequently detected by human HTLV-III antibodies from AIDS patients. Immunoprecipitation of the gp41, gp120 and gp160 proteins occurred when using a lentil

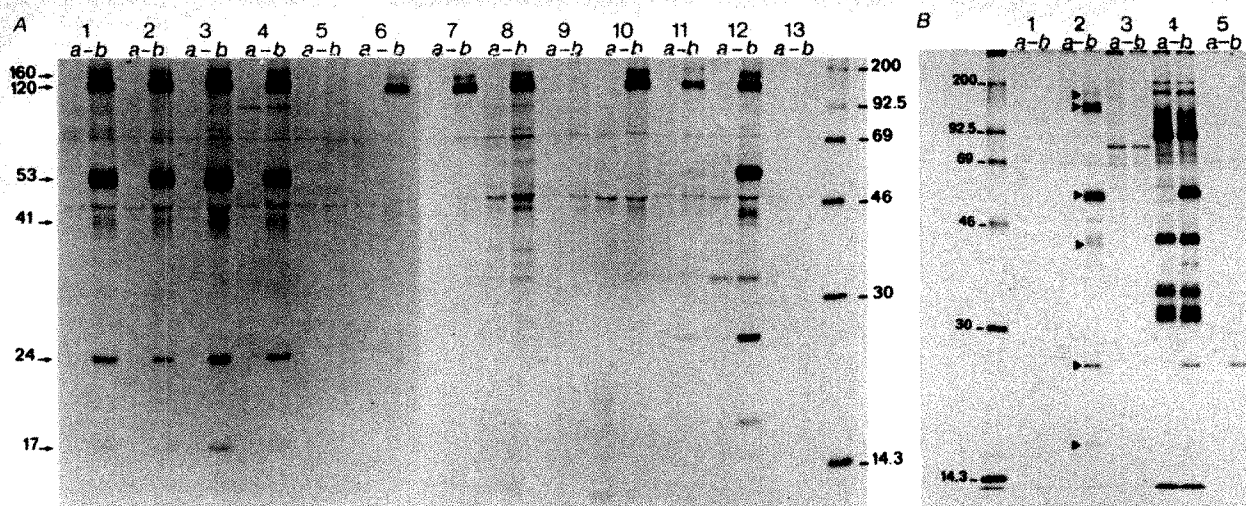


Fig. 1 A, ^{35} S-cysteine-labelled antigens precipitated from HTLV-III-negative H9 cells (a) and HTLV-III-infected H9 cells (b) by serum samples from asymptomatic haemophiliacs (lanes 1-5, 9-11). Lane 12: a positive control AIDS patient. Lane 13: a negative control (healthy laboratory worker). Lanes 6 and 7: asymptomatic haemophiliacs who developed AIDS within 3-6 months. Lane 8: a haemophiliac AIDS patient. Numbers on the left refer to proteins discussed in the text; numbers on the right refer to molecular weight markers ($\times 10^{-3}$). B, ^{35} S-cysteine-labelled antigens precipitated from HTLV-III-negative cells (a) and HTLV-III-infected H9 cells (b). Serum samples were from: lane 1, a negative control (same as lane 13 in A); lane 2, a positive control AIDS patient (same as lane 12 in A); lane 3, a normal rabbit; lane 4, a reference rabbit antiserum to disrupted HTLV-III³; lane 5, a monoclonal antibody to HTLV-III p24. Numbers on the left refer to molecular weight markers ($\times 10^{-3}$).

lectin-bound, glycoprotein-enriched cell lysate fraction as described earlier, which suggests that these antigens are glycoprotein in nature¹⁰. Seventeen of the serum samples failed to react by either IMI or RIP-SDS/PAGE. Of the 33 serum samples that were positive for antibody to HTLV-III-related glycoproteins, 23 also proved positive for antibody to virus core antigens. p24 is the major virion core protein and p53 is presumed to be the gag gene-encoded polypeptide because, as Gallo and his co-workers¹⁻⁴ and workers in our laboratory (T. H. Lee and M.E., manuscript in preparation) have found, it is readily precipitated with reference antiserum to disrupted HTLV-III. Some haemophiliac samples precipitated a protein of 17,000 molecular weight from H9/HTLV-III cells; this protein is also precipitated by a reference rabbit antiserum to HTLV-III core proteins.

The 50 haemophiliacs tested included three who developed AIDS. All three tested positive by both procedures. Patients who tested positive ranged in age from 4 to 57 yr and included patients with haemophilia A (32 of 44) and haemophilia B (1 of 6). When divided according to the time of sample collection, 18 of 34 (53%) of individuals samples before 1984 were positive whereas 15 of 16 (94%) of those studied during 1984 were positive (one-sided P value ≤ 0.002).

Evidence has been accumulating which suggests that a member of the human T-cell leukaemia retrovirus (HTLV) family is involved in the pathogenesis of AIDS^{1-5,11-17}. Other retroviruses have been linked with naturally occurring immunosuppression and leukaemia in cats¹⁸, and a virus closely related to HTLV

has been associated with leukaemias and lymphoproliferative diseases that have occurred as part of an AIDS-type outbreak in macaque monkeys⁸.

We have previously performed IMI on serum samples of haemophiliacs to test for the presence of antibodies to membrane antigens of cells infected with HTLV-I (HTLV-MA)^{9,11,19}. Haemophiliacs were also tested for antibodies to cytomegalovirus (CMV) and Epstein-Barr virus (EBV)²⁰. Sixty-nine per cent of haemophiliacs had antibodies to EBV and 42% had antibodies to CMV. Approximately 12% of 172 asymptomatic haemophiliacs from four cities tested positive for HTLV-MA; specificity was confirmed with RIP-SDS/PAGE⁹. Because a significant proportion of the haemophiliacs from New York had evidence of antibodies to HTLV-MA before 1980, when AIDS was first described in the United States, it seemed likely that some had been exposed to a conventional strain of HTLV rather than to HTLV-III, the strain that seems to be the aetiological agent of AIDS²¹.

The present results suggest that exposure to HTLV-III is widespread in asymptomatic haemophiliacs. Earlier studies indicated that antibodies to HTLV-I and HTLV-II envelope gene glycoproteins are associated with the development of AIDS^{11,15,22}, presumably because the antibodies are at least partially cross-reactive with HTLV-III proteins¹⁻⁴. Both HTLV-I and AIDS, which is thought to be caused by HTLV-III, seem to be transmitted by blood transfusions and blood products^{15,23-26}. In a previous study, two of three haemophiliacs with AIDS had antibodies to HTLV-MA of type I⁹ and in the

Table 1 Presence of antibodies to HTLV-III in haemophiliacs and healthy laboratory workers

Category	No. tested	HTLV-MA	gp41/gp120/gp160	p24/p53
Haemophilia A	44	32 (73)	32 (73)	23 (52)
Haemophilia B	6	1 (17)	1 (17)	0 (0)
1984	16	15 (94)	15 (94)	13 (81)
Pre-1984	34	18 (53)	18 (53)	10 (29)
Asymptomatic	47	30 (64)	30 (64)	23 (49)
Haemophilia with AIDS	3	3 (100)	3 (100)	0 (0)
Total haemophiliacs	50	33 (66)	33 (66)	23 (46)
Healthy laboratory workers	29	0 (0)	0 (0)	0 (0)

Antibodies to HTLV-MA were determined by IMI; those to gp41/gp120/gp160 and p24/p53 were determined by RIP-SDS/PAGE.

present study two asymptomatic haemophiliacs who subsequently developed AIDS had antibodies to HTLV-III.

The observation that the proportion of asymptomatic haemophiliacs with antibodies to HTLV-III may have increased from 1983 to 1984 deserves further study using sequential samples from the same individuals. As the incubation period for the development of AIDS following blood transfusion has been estimated to range from 10 months to 4 yr²⁶, further studies are needed to evaluate the impact of this observation. Antibody positivity seems to be an indicator of viral infection in most healthy and leukaemic individuals who were naturally exposed to HTLV-I²⁷⁻²⁹ as well as in AIDS patients and healthy male homosexuals exposed to HTLV-III¹⁻⁴. However, it is possible that a significant proportion of asymptomatic haemophiliacs might be exposed only to inactivated HTLV-III rather than to live virus, owing to the manufacturing process involved in the preparation of commercial factor VIII concentrate. In addition,

it is clear that those individuals exposed to factor IX preparations are also at risk for the development of antibodies to HTLV-III. Further studies are needed to determine whether the presence of antibodies to HTLV-III indicates immunization, infection or an asymptomatic carrier state. In addition, the relationship between HTLV-III seropositivity and immunological dysfunction, which has been shown to be widespread among apparently healthy haemophiliacs^{30,31}, requires investigation.

We thank Dr R. C. Gallo for the reference cell cultures and the reference antisera. This research was supported by National Research Service Award ST 32CA9382 (L.W.K.), Association pour le Développement de la Recherche sur le Cancer, Fondation pour la Recherche Médicale, and Philippe Foundation (F.B.), NIH grants AI21161 (J.L.S.) and CA37466 and CA18216 (M.E.). J.L.S. is an established investigator of the American Heart Association.

Received 30 August; accepted 26 September 1984.

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Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection

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Interstitial pneumonia linked with reactivation of latent human cytomegalovirus due to iatrogenic immunosuppression can be a serious complication of bone marrow transplantation therapy of aplastic anaemia and acute leukaemia¹. Cellular immunity plays a critical role in the immune surveillance of inapparent cytomegalovirus infections in man and the mouse¹⁻⁷. The molecular basis of latency, however, and the interaction between latently or recurrently infected cells and the immune system of the host are poorly understood. We have detected a so far unknown antigen in the mouse model. This antigen is found in infected cells in association with the expression of the herpesvirus 'immediate early' genes and is recognized by cytolytic T lymphocytes (CTL)⁸. We now demonstrate that an unexpectedly high proportion of the CTL precursors generated *in vivo* during acute murine cytomegalovirus infection are specific for cells that selectively synthesize immediate early proteins, indicating an immunodominant role of viral non-structural proteins.

According to the static state hypothesis of latency⁹, it is assumed that viral progeny are not produced because the transcription process of the virus is interrupted reversibly at an early stage in the cycle of virus multiplication. In herpes virus infections of permissive cells *in vitro*, analysed in detail for herpes simplex viruses^{10,11}, viral protein synthesis is coordinately regulated and starts with the expression of the 'immediate early' (IE) genes, which are transcribed by host RNA polymerase II. The IE proteins exert important control functions as exemplified by their ability to act as transcriptional activators^{12,13} and they need to be present throughout the replication cycle of herpes-

Table 1 Determination of the IL-CTLp frequency by LDCC

Dose of lectin ($\mu\text{g ml}^{-1}$)	IL-CTLp frequency	No. of IL-CTLp per 10^6 lymphocytes (95% confidence limits)	pMC*
Con A	5	171 (126-215)	0.23
	10	359 (217-502)	0.11
	20	383 (270-495)	0.50
PHA	5	179 (124-234)	0.24
	10	105 (71-140)	0.52
	20	132 (93-171)	0.24

BALB/c (*H-2^d* haplotype) mice were infected at the left hind footpad with 10^5 PFU of tissue-culture propagated MCMV (ATCC VR-194 Smith strain) and 8 days later lymphocytes derived from the draining popliteal lymph nodes were tested for the frequency of activated CTLp in a limiting dilution assay²⁰. In brief, graded numbers of lymphocytes were cultivated for 6 days in 96-well round-bottomed microtitre plates with no addition of viral antigen using medium MEM- α (Gibco) supplemented with 10% vol. fetal calf serum and 20% vol. interleukin-2 containing supernatant. Starting the dilution series with 1×10^5 lymphocytes, 7 log₂ dilution steps were performed with 24 replicate microcultures each. To detect all CTL clones irrespective of their antigen-specificity, the LDCC assay was applied. Various doses of the lectins Con A (Pharmacia) or PHA (Flow Laboratories) were added to 10^3 ⁵¹Cr-labelled, noninfected MEF and CTL at the beginning of a standard 3-h ⁵¹Cr release assay. Precursor frequencies were estimated from the proportion of nonresponding microcultures using the minimum χ^2 (MC)³² and maximum likelihood (ML)³³ methods. Only the results of the MC evaluation are shown.

* Probability calculated by MC method. According to the proposition of Taswell³² an estimate was accepted for a probability of $P > 0.05$ and a ML-MC deviation of $<10\%$.

viruses to permit transcription of the 'early' and 'late' genes¹⁴. Interference with viral transcription at the stage of the expression of IE genes may be involved in establishing viral latency; hence many efforts have been made to clone and map the IE regions of herpesvirus genomes, including those of human^{15,16} and murine cytomegalovirus (MCMV)¹⁷⁻¹⁹. In particular, re-expression of IE genes must be one of the first events in virus reactivation from latency. Selective synthesis of IE proteins leads

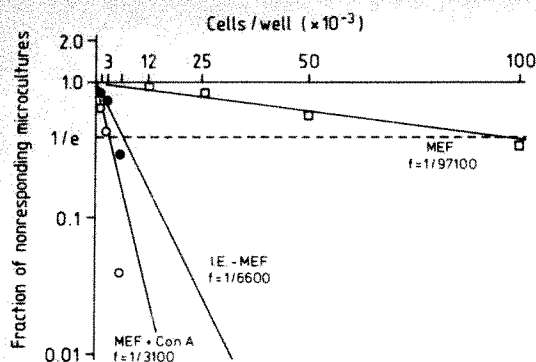


Fig. 1 High frequency of LYDIEA-specific IL-CTLp. CTL were generated by *in vitro* interleukin-mediated expansion of *in vivo* activated interleukin-receptive CTLp (IL-CTLp) as outlined in Table 1. Second-passage mouse embryo fibroblasts (MEF) were used as a source of target cells for the ^{51}Cr -release assay. To determine the negative or positive reference frequencies, noninfected MEF were used either in the absence of lectin or with addition of $20\text{ }\mu\text{g ml}^{-1}$ Con A, respectively. Infected target cells after selective expression of immediate early genes (IE-MEF) served to determine the frequency of IL-CTLp specific for the immediate early antigen (LYDIEA) of MCMV. Details of the limiting dilution assay are given in Table 1 and details of the target cell preparation in Table 2.

to the expression of an antigen on the cell surface⁸. This antigen is recognized by CTL and was therefore designated lymphocyte-detected IE antigen (LYDIEA). LYDIEA-specific CTL represented a distinct subset, separate from those CTL recognizing viral structural antigens⁸. The mere fact, however, that during MCMV infection LYDIEA-specific CTL are generated did not allow definite conclusions about the relative significance of the immune response to LYDIEA in comparison with the response to other antigens specified by MCMV.

The solution of two experimental problems was a prerequisite for attempts to evaluate the significance of LYDIEA. First, *in vivo* primed CTL precursors (CTLp) can be detected only after clonal expansion *in vitro* by testing their cytolytically active progeny. This expansion has to be performed in the absence of antigen because restimulation *in vitro* may favour or disfavour the growth of CTLp specific for certain antigens and thereby cause a distortion of the numerical *in vivo* representation of specificities. The expansion of interleukin-receptive CTLp (IL-

CTLp) by interleukin solves this problem as that procedure selects cells only on the basis of differentiation²⁰.

The second problem arises from the fact that progeny CTL are usually detected by their ability to recognize target cells carrying the relevant antigens. This recognition requires sufficient amounts of antigen to be present at the cell membrane²¹. Selective and sufficient expression of LYDIEA can be achieved⁸, but it is not certain in a particular experiment whether each of the other viral antigens is also expressed sufficiently. Therefore, to detect all IL-CTLp generated during MCMV infection regardless of their antigen-specificity, the lectin-dependent cellular cytotoxicity (LDCC) assay was applied. In this assay the T-cell mitogenic lectins concanavalin A (Con A) and phytohaemagglutinin (PHA) are used to bypass the requirement for specific interaction between CTL and target cells^{22,23}. The highest frequency of IL-CTLp was measured when ConA was added at doses of $10\text{--}20\text{ }\mu\text{g ml}^{-1}$ (Table 1). The fact that the corresponding frequency in unprimed mice ranged from 1 in 70,400 to 1 in 91,400 lymphocytes (data not shown) proved that the CTL detected in the lectin-dependent cellular cytotoxicity (LDCC) assay were derived from *in vivo* sensitized precursors. This condition was then used to assess the total frequency of IL-CTLp.

The results of a representative limiting dilution assay are shown in Fig. 1 and those of an independent second experiment summarized in Table 2. The frequency of LYDIEA-specific IL-CTLp present in a draining popliteal lymph node was found to be 1 in 6,600 lymphocytes (Fig. 1) or 1 in 6,900 lymphocytes, respectively (Table 2). This determination was carried out on target cells that selectively expressed high amounts of IE proteins. The selective transcription of IE genes in target cells was achieved by infection in the presence of the translation inhibitor cycloheximide that led to an accumulation of IE RNA. The subsequent release from cycloheximide and further cultivation in the presence of the transcription inhibitor actinomycin D then resulted in an enhanced synthesis of IE proteins and simultaneously prevented further viral transcription^{8,19}.

During noninterrupted (productive) infection, the synthesis of IE proteins is followed by the synthesis of so-called early proteins and, after viral DNA replication, of late proteins which include the major viral structural proteins^{10,24}. Among those especially the glycoproteins of the virion envelope should contribute significantly to the antigenicity of infected cells. Nevertheless, when tested on cells corresponding to the late

Table 2 IL-CTLp frequencies as determined by antigen-specific or lectin-dependent cellular cytotoxicity

Target cell (virus dose; inhibitors)	IL-CTLp frequency	No. of IL-CTLp per 10^6 lymphocytes (95% confidence limits)	pMC
MEF (—; CH-Act.D)	1/166,400	6 (4–8)	0.35
MEF + Con A (—; CH-Act.D)	1/2,700	371 (226–516)	0.27
IE-MEF (0.05; CH-Act.D)	1/6,900	146 (97–195)	0.16
Late-MEF (0.05; -Act.D)	1/11,300	89 (60–118)	0.24
UV-MCMV-MEF (0.05; CH-Act.D)	1/85,300	12 (8–16)	0.35
UV-MCMV-MEF (0.5; CH-Act.D)	1/44,300	23 (12–33)	0.16
UV-MCMV-MEF (5; CH-Act.D)	1/14,900	67 (48–86)	0.08

Second-passage MEF were used as target cells throughout. Infection was performed under centrifugal enhancement conditions³⁴ ($800g$ for 30 min) with the indicated virus dose of infection that is given in plaque-forming units (PFU) per cell as determined in a conventional plaque titration assay. The centrifugation enhances the efficiency of infection by 20- to 80-fold and thus a virus dose of 0.05 PFU corresponds with an effective multiplicity of about 1–4 PFU per cell. At a multiplicity of 100–400 PFU per cell target cells started to disintegrate and therefore the application of higher doses was precluded. For the preparation of IE targets and the appropriate controls, infection and 3 h of cultivation were performed in the presence of cycloheximide (CH, $50\text{ }\mu\text{g ml}^{-1}$). Thereafter, CH was washed out and actinomycin D (Act.D, $5\text{ }\mu\text{g ml}^{-1}$) was added. Only for the preparation of the late-MEF target, infection was carried out in the absence of CH and viral replication was allowed to proceed for 20 h until transcription was stopped by addition of actinomycin D. To test lectin-dependent cytotoxicity, Con A was added during the effector phase at a final dose of $20\text{ }\mu\text{g/ml}$. For mock-infection of MEF, partially purified stock virus ($2 \times 10^8\text{ PFU ml}^{-1}$)²⁰ was diluted with phosphate-buffered saline to a final concentration of $1 \times 10^7\text{ PFU ml}^{-1}$ and then exposed as an 1-mm layer to a 10-min irradiation with a 90 W UV-illuminator at 254 nm. The distance between specimen surface and ultraviolet-screen was adjusted to give an intensity of $30\text{ J m}^{-2}\text{ s}^{-1}$. To control the effect of irradiation on viral protein synthesis³⁵, MEF were infected with the highest dose of virus used in the experiments (5 PFU equivalents per cell) and IE protein synthesis was enhanced by inhibitor treatment. Monoclonal antibody 6/58/1 (to be described elsewhere) was then used to detect IE nuclear antigen by indirect immunofluorescence. Irradiation of virus for 2.5 min ($4,500\text{ J m}^{-2}$) reduced the number of IE antigen positive cells to less than 1% and after 5 min of irradiation viral protein synthesis was abolished in all cells.

stage of viral replication, the frequency of IL-CTLp was only 1 in 12,800 lymphocytes (not depicted in Fig. 1) or 1 in 11,300 lymphocytes, respectively (Table 2).

In addition to *de novo* synthesized antigens, virion proteins or glycoproteins inserted into the cell membrane during the process of penetration can directly account for an antigenicity of MCMV-infected cells. This has been demonstrated previously by infection of cells in the continuous presence of actinomycin D or cycloheximide⁶. Different treatment, mock-infection with UV-inactivated virus, still permits the synthesis of cellular proteins which might be induced by viral structural proteins and could also contribute to target formation. This control is therefore indicated to discriminate between target antigens induced by the IE activity of the viral genome and those antigens whose expression does not require IE protein synthesis. When mock-infection of targets was performed with a dose of ultraviolet-inactivated virus equivalent to the dose of infectious virus applied for preparing the IE target, the frequency was only 1 IL-CTLp in 85,300 lymphocytes (Table 2). This result proved that the high frequency of 1 IL-CTLp in 6,900 lymphocytes, detected with the IE target, was indeed due to the expression of an IE antigen and thus referred to LYDIEA-specific IL-CTLp. This conclusion is further confirmed by the finding that in the absence of viral genome-induced protein synthesis even a hundred-fold increase in the virus dose did not allow to detect the number of IL-CTLp that could be detected with the IE target or with the productively infected target (Table 2).

The total frequency of IL-CTLp in these experiments was found to be 1 in 3,100 lymphocytes (Fig. 1) or 1 in 2,700 lymphocytes, respectively (Table 2). Hence, based on the determination of the total IL-CTLp frequency, the LYDIEA-specific subset comprised 40–50% of all IL-CTLp generated during infection.

The finding that such a substantial proportion of the IL-CTLp recognize an antigen expressed during the IE phase of viral replication was unexpected because transcription at that stage is restricted to only few genes, while extensive transcription from the whole genome occurs at later stages¹⁹.

Three possibilities might explain the high frequency of LYDIEA-specific IL-CTLp: First, the primary T cell specificity repertoire of BALB/c mice may already contain a high relative number of precursors specific for this antigen. There is no approach to test this possibility as limiting dilution experiments have revealed an extremely low frequency of primary CTLp specific for MCMV²⁰ or herpes simplex virus²⁵ and that is why a reliable comparison between particular antigens is not feasible.

Second, the expression of antigens may be different in the tissues of the infected animal than predicted from the productive infection of embryonic fibroblasts *in vitro*. Since in the immunocompetent host MCMV does not replicate at the site of intraplantar infection, whereas after immunosuppression fibroblasts of the footpad tissue are productively infected (manuscript in preparation), the immune system is involved in terminating the *in vivo* viral replication at an early stage. This could imply a preferential sensitization of LYDIEA-specific CTLp and explain the high proportion of LYDIEA-specific IL-CTLp in the draining lymph node. We are now testing the amount of IE gene expression by *in situ* hybridization using cloned viral DNA fragments¹⁹.

Third, the IE antigen may be highly immunogenic. Its molecular identity has first to be defined in order to evaluate this alternative. At present it is known that the expression of LYDIEA requires transcription and translation⁸ and coincides with the synthesis of a phosphorylated but not glycosylated protein of 89,000 apparent relative molecular mass. This protein and its post-translational modification products are the dominant IE proteins located in the nucleus and cytoplasm of the infected cell (G. M. Keil *et al.*, in preparation). Since recognition by CTL must be considered as a *prima facie* evidence for surface localization of an antigen, the actual relationship between these serologically detectable intracellular proteins and the antigen LYDIEA requires further analysis. The presence of

nonglycosylated viral proteins, located predominantly in the nucleus and cytoplasm, coincides with the recognition of infected cells by CTL also in other virus infections such as Epstein-Barr virus²⁶, simian virus 40^{27,28} and influenza virus^{29–31}. In this context it has been suggested that processed products rather than the intact protein may represent the actual antigen³¹ or that the intracellular protein may modify other viral or cellular products²⁹.

Irrespective of the so far unknown molecular identity of LYDIEA, the requirement of IE proteins for the synthesis of viral structural proteins during acute infection or after reactivation from latency raises the possibility of a physiological role of LYDIEA-specific CTL in the surveillance of viral latency. LYDIEA-specific memory-CTL which are present in latently infected mice⁸ could be activated as soon as IE genes are expressed and eliminate recurrently infected cells before the assembly of progeny virions.

This work was supported by two grants to U.H.K. of the Deutsche Forschungsgemeinschaft, SFB 120 and Ko 571/8. We thank Ms A. Lüske for technical assistance.

Received 17 April; accepted 11 September 1984.

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Phorbol ester and diacylglycerol mimic growth factors in raising cytoplasmic pH

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There is now good evidence that cytoplasmic pH (pH_i) may have an important role in the metabolic activation of quiescent cells^{1,2}. In particular, growth stimulation of mammalian fibroblasts leads to a rapid increase in pH_i (refs 3–6), due to activation of a Na^+/H^+ exchanger in the plasma membrane⁴, and this alkalization is necessary for the initiation of DNA synthesis⁷. However, the mechanism by which mitogens activate the Na^+/H^+ exchanger to raise pH_i is not known, although an increase in cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) has been postulated as the primary trigger^{8–10}. We

now present data suggesting that the Na^+/H^+ exchanger is set in motion through protein kinase C, a phospholipid- and Ca^{2+} -dependent enzyme normally activated by diacylglycerol produced from inositol phospholipids in response to external stimuli^{11,12}. Using newly developed pH microelectrodes and fluorimetric techniques, we show that a tumour promoting phorbol ester and synthetic diacylglycerol, both potent activators of kinase C (refs 12–15), mimic the action of mitogens in rapidly elevating pH_i in different cell types. Furthermore, we demonstrate that, contrary to previous views, an early rise in $[\text{Ca}^{2+}]_i$ is not essential for the activation of Na^+/H^+ exchange and the resultant increase in pH_i . Finally, we suggest that an alkaline pH_i shift, mediated by Na^+/H^+ exchange, may be a common signal in the action of those hormones which elicit the breakdown of inositol phospholipids.

Human foreskin fibroblasts (HF cells), HeLa cells and mouse N1E-115 neuroblastoma cells, grown on rectangular glass coverslips, were loaded with the fluorescent pH indicator 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) by uptake of its membrane-permeable ester¹⁶, and the pH_i -dependent emission intensity from the cells was continuously monitored as described previously^{4,17}. In addition, pH_i was directly measured in large N1E-115 cells by means of a novel type of pH-sensitive intracellular microelectrode (see Fig. 1 legend).

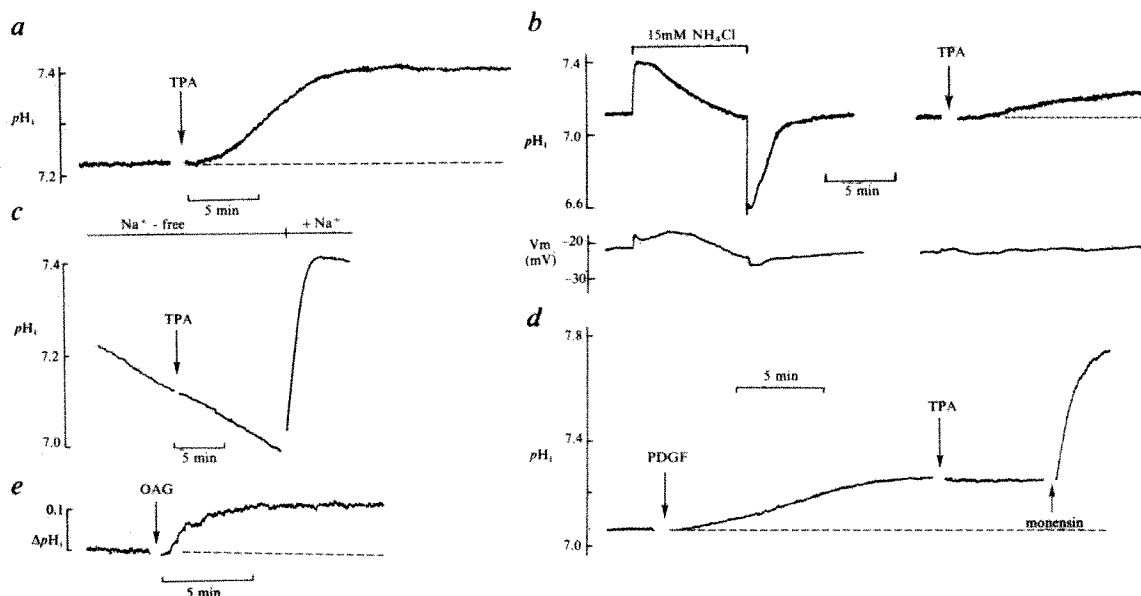


Fig. 1 *a*, Time course of increase in pH_i induced by TPA (200 ng ml^{-1}) in mouse N1E-115 neuroblastoma cells. Subconfluent cultures, attached to a glass coverslip, were deprived of serum for 24 h and loaded with BCECF as described previously⁴. The cells were incubated in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM, pH 7.40) without phenol red and pH_i -dependent fluorescence was measured at 506–532 nm using a Perkin Elmer model 3000 fluorescence spectrometer. The fluorescence of BCECF varies linearly with pH over the range 6.4–7.6 (ref. 17). Temperature was maintained at 32°C. Similar TPA-induced shifts in pH_i were observed in serum-deprived HeLa cells (subconfluent) and HF cells (confluent). *b*, Direct measurement of pH_i and membrane potential (V_m) in large N1E-115 cells (diameter $> 40 \mu\text{m}$), penetrated by a pH-sensitive microelectrode and a voltage-measuring microelectrode, showing rapid recovery of pH_i from a NH_4^+ -induced acid load, and TPA-induced rise in pH_i . pH_i regulation in N1E-115 cells, as in HF cells and HeLa cells, is almost entirely mediated by amiloride-sensitive Na^+/H^+ exchange (refs 4, 17, 32 and W.H.M., unpublished results). The method for constructing pH-sensitive microelectrodes is described below. Solutions were exchanged by rapid perfusion. Other experimental details are essentially similar to those described previously^{33,34}. Final TPA concentration, 200 ng ml^{-1} . External pH 7.40. Temperature, 35°C. *c*, Lack of effect of TPA on pH_i in BCECF-loaded N1E-115 cells maintained in Na^+ -free medium. Na^+ was replaced by bis(2-hydroxyethyl)dimethylammonium. Cells rapidly acidify in Na^+ -free medium, but pH_i immediately recovers after re-addition of Na^+ (see refs 4, 17, 32). *d*, Effects of PDGF (40 ng ml^{-1}), TPA (200 ng ml^{-1}) and monensin ($5 \mu\text{M}$) on pH_i in BCECF-loaded culture of quiescent HF cells. Insulin was present at $5 \mu\text{g ml}^{-1}$ to potentiate PDGF-induced pH_i shift (insulin alone has no effect on pH_i ; ref. 4). Human PDGF ($>95\%$ pure) was added in the presence of $100 \mu\text{g ml}^{-1}$ bovine serum albumin (essentially fatty acid free). *e*, Effect of synthetic OAG ($50 \mu\text{g ml}^{-1}$) on pH_i in BCECF-loaded N1E-115 cells. OAG stock solution in dimethyl sulphoxide (DMSO) was diluted to 1% DMSO with DMEM + $50 \mu\text{g ml}^{-1}$ bovine serum albumin (fatty acid free). A 250- μl aliquot of this OAG solution was then briefly sonicated under N_2 and immediately added to the cells. The final DMSO concentration was 0.1%, which alone had no effect.

Methods: Construction of pH-sensitive microelectrodes: An 8-mm length of pH-sensitive glass tubing (outer diameter 1.0 mm; inner diameter 0.5 mm; Clark Electromedical Instruments) was placed in the centre of a 5-cm piece of insulating lead glass tubing (outer diameter 1.6 mm; wall thickness $<0.15 \text{ mm}$). The coaxial tubes were pulled into two microelectrodes using a horizontal micropipette puller. About 50–60% of the electrodes then contains a sealed pH-sensitive tip with an outer diameter of $<0.5 \mu\text{m}$. Electrodes were first filled with methanol by boiling under reduced pressure at room temperature. Methanol was then replaced with 0.1 M HCl by diffusion. Subsequently, the electrode tips were immersed into a chromic acid cleaning solution for 12–24 h before experimentation. Electrodes were tested for giving a Nernstian response to pH changes (54–58 mV per pH unit) and having resistance of $1\text{--}2 \times 10^9 \Omega$. The speed of response to a sudden pH change of 1 unit was about 15–20 s. Selected electrodes could be stored for a few days (tips in chromic acid) before obvious deterioration. The construction of this novel type of pH-sensitive electrode was found to be much easier than that of the common 'Thomas type' pH microelectrodes³⁵. Further details will be described elsewhere.

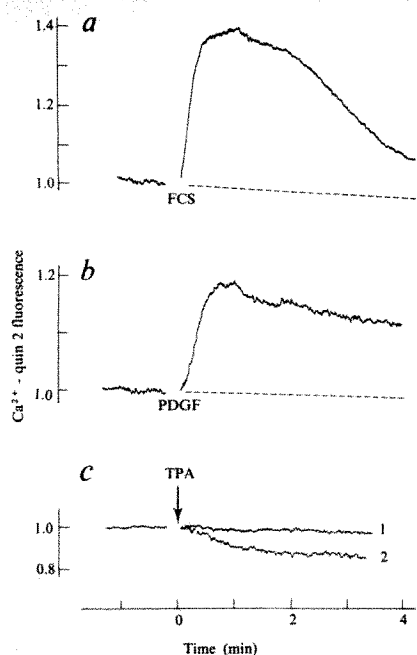


Fig. 2 Rapid changes in $[Ca^{2+}]_i$ in HF cells after addition of FCS (10%) (a), PDGF (40 ng ml⁻¹) (b) and TPA (c; trace 1, 50 ng ml⁻¹; trace 2, 200 ng ml⁻¹) at time zero. Quiescent HF monolayers were loaded with quin-2 by incubating them in DMEM containing 50 μ M quin-2 acetoxymethyl ester (Amersham) for 30 min at 37°C and $[Ca^{2+}]_i$ -dependent fluorescence was continuously measured at 339–500 nm (temperature, 32°C). Signals were calibrated essentially as described elsewhere^{26,36}. Resting value of $[Ca^{2+}]_i$ is estimated at 140 nM, while peak values of the FCS and PDGF traces correspond to ~400 and 300 nM $[Ca^{2+}]_i$, respectively.

by the magnitude of the transmembrane Na^+ and H^+ gradients, as pH_i can be increased still further by addition of the Na^+/H^+ ionophore monensin (Fig. 1d). From these results we conclude that TPA, like growth factors^{4–6}, raises pH_i by activating the otherwise quiescent Na^+/H^+ exchanger in the plasma membrane.

If activation of Na^+/H^+ exchange is mediated by kinase C, an alkaline pH_i shift may be expected after addition of an unsaturated diacylglycerol such as 1-oleoyl-2-acetyl-glycerol (OAG; refs 14, 15). In physiological conditions, 1,2-diacylglycerol is produced from hydrolysis of inositol phospholipids and serves as the endogenous activator of protein kinase C^{11,12}. As illustrated in Fig. 1e, addition of 50 μ g ml⁻¹ OAG to N1E-115 cells does indeed induce a rapid alkaline shift in pH_i of at least 0.1 unit. A similar shift was observed in OAG-treated HeLa cells. Other lipid degradation products such as phosphatidic acid and arachidonic acid, a precursor of prostaglandins, were unable to raise pH_i . Furthermore, indomethacin (5 μ M), a potent inhibitor of prostaglandin synthesis, did not affect the pH_i shifts induced by either TPA or PDGF. Various lipid-interacting drugs, such as the phenothiazines, are potent inhibitors of protein kinase C^{20,21}. We found that trifluoperazine (TFP) at 10 μ M produced ~50% inhibition of both the PDGF- and TPA-evoked pH_i shifts in HF cells without affecting basal pH_i levels. (At higher TFP doses the cytoplasm gradually acidified, presumably due to a nonspecific membrane perturbation and consequent H^+ influx.) The above results, together with the fact that growth factors are potent inducers of inositol lipid breakdown^{22,23}, strongly suggest that protein kinase C is responsible for the activation of Na^+/H^+ exchange by mitogens and phorbol ester.

Although the Ca^{2+} ionophore A23187 is incapable of elevating pH_i in fibroblasts⁴, the possibility nevertheless remains that a rapid increase in $[Ca^{2+}]_i$ may have some critical role in Na^+/H^+ exchange activation, as postulated by others¹⁰. We therefore used the intracellularly-trapped fluorescent dye quin-2 (refs 24,

Table 1 Effects of TPA on pH_i

Cell type	Resting pH_i (mean $\pm$ s.e.)	ΔpH_i (relative to control)
HF	7.05 $\pm$ 0.02 (n = 12)	0.16 $\pm$ 0.03 (n = 5)
HeLa	7.18 $\pm$ 0.04	0.17 $\pm$ 0.02 (n = 7)
N1E-115	7.24 $\pm$ 0.03	0.15 $\pm$ 0.02 (n = 8)

Cells were loaded with BCECF and assayed for TPA-induced shifts in pH_i (ΔpH_i) by the K^+ -nigericin calibration method as detailed elsewhere^{4,17}. HF and N1E-115 cells were maintained in serum-free DMEM for 24 h and HeLa cells for 4 h before experimentation. TPA concentration, 200 ng ml⁻¹. External pH , 7.30–7.40. Temperature, 30–32°C. The number of experiments is given in parentheses.

25) to monitor $[Ca^{2+}]_i$ after activation of quiescent HF and N1E-115 cells. Figure 2a, b illustrates typical results from HF cells, the details of which are presented elsewhere²⁶, and shows that fetal calf serum (FCS) and PDGF immediately raise $[Ca^{2+}]_i$ two- to threefold above its resting level of about 140 nM. This mitogen-induced Ca^{2+} signal is initiated without a detectable lag period and peaks within 1 min before slowly declining towards a new steady level. The Ca^{2+} response is not prevented by removal of external Ca^{2+} (ref. 26), indicating that FCS and PDGF act by releasing Ca^{2+} from intracellular stores, probably due to the rapid formation of inositol 1,4,5-trisphosphate²⁷. A qualitatively similar Ca^{2+} signal was observed in serum-stimulated N1E-115 cells (not shown).

By contrast, TPA, at concentrations that evoke a significant pH_i shift, completely fails to raise $[Ca^{2+}]_i$ above basal levels in HF cells (Fig. 2c). At 200 ng ml⁻¹, TPA even induces a slight fall in $[Ca^{2+}]_i$. The apparent inability of TPA to elevate $[Ca^{2+}]_i$ agrees with previous findings in quin-2-loaded lymphocytes²⁴, neutrophils²⁸ and platelets²⁹. These findings demonstrate that although growth factors are Ca^{2+} -mobilizing hormones, an early rise in $[Ca^{2+}]_i$ is not essential for the activation of Na^+/H^+ exchange by external stimuli, and they reinforce the notion that the Na^+/H^+ exchanger is set in motion mainly through activated kinase C. Thus, the simplest and most attractive explanation for our findings is that protein kinase C directly phosphorylates the Na^+/H^+ exchanger on its cytoplasmic face and thereby increases its apparent affinity for intracellular H^+ (see ref. 4).

The results reported here raise the interesting possibility that cytoplasmic alkalization, mediated by Na^+/H^+ exchange, is not restricted to the action of growth factors and tumour-promoting phorbol esters, but that it may commonly occur in response to those hormones and other surface stimulants which trigger the breakdown of inositol phospholipids and thereby activate kinase C. There is indeed some evidence to support this notion: in typical model systems of hormone-induced inositol lipid turnover, such as platelets and neutrophils, the pH_i rises rapidly on activation by physiological stimuli^{30,31}. Further work will be needed to explore the ubiquity of cytoplasmic alkalization in hormone action and to identify the pH_i -dependent responses in different target cells.

We thank Roger Y. Tsien for gifts of BCECF-ester and OAG, Carl-Henrik Heldin for providing pure PDGF, Lilian Joosen for technical assistance and Dr M. J. Berridge for stimulating discussions. This work was supported by grants from the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

Received 15 May; accepted 11 September 1984.

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Stepwise enzymatic dephosphorylation of inositol 1,4,5-trisphosphate to inositol in liver

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Many receptors for hormones, neurotransmitters and other signals cause hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) and effect a rise in cytosolic Ca²⁺ concentration¹⁻⁷. The inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) liberated during PtdIns(4,5)P₂ breakdown seems to serve as a second messenger that activates the release of Ca²⁺ from a nonmitochondrial intracellular compartment⁷⁻¹². As expected if it is an important intracellular messenger, Ins(1,4,5)P₃ is relatively rapidly degraded, both within stimulated cells^{13,14} and when added to homogenates of blowfly salivary gland¹⁵ or to permeabilized, but not intact, hepatocytes¹⁰. Here we report that the dephosphorylation reactions responsible for the conversion of Ins(1,4,5)P₃ to free inositol in rat liver are catalysed by two or more enzymes, and that these reactions are distributed between the plasma membrane and cytosol. The Ins(1,4,5)P₃ 5-phosphatase and inositol 1-phosphatase (Ins(1)P) phosphatase of liver appear similar to enzymes described previously in erythrocytes¹⁶ and brain¹⁷.

Rat liver homogenates were incubated with small concentrations of ³²P-labelled inositol phosphates in conditions of ionic composition and pH chosen to approximately mimic those of the cytosol of stimulated cells (for details, see legend to Table 1). Ins(1,4,5)P₃ (labelled in its 4- and 5-phosphate groups), inositol-1,4-bisphosphate (Ins(1,4)P₂) (labelled in its 4-phosphate) and Ins(1)P were rapidly degraded during such incubations, with hydrolysis of Ins(1,4,5)P₃ being the most rapid reaction (Table 1). High-speed centrifugation sedimented a large proportion of the Ins(1,4,5)P₃ phosphatase activity and left most of the Ins(1,4)P₂ phosphatase and Ins(1)P phosphatase activity in the soluble fraction (Table 1). Analysis of the products of Ins(1,4,5)P₃ hydrolysis^{16,18} by a plasma membrane/Golgi-rich membrane fraction recovered from a density gradient separation (as in Fig. 1) showed selective removal of the 5-phosphate (data not shown).

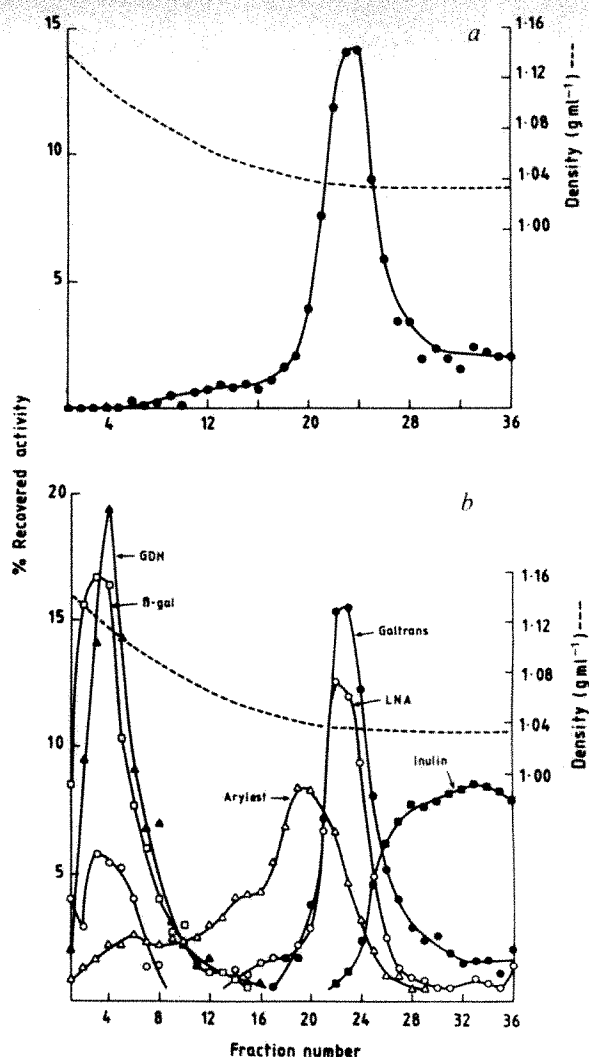


Fig. 1 Distribution of Ins(1,4,5)P₃ 5-phosphatase and various marker enzymes after density gradient centrifugation of a rat liver nuclei-free homogenate. *a*, The distribution of Ins(1,4,5)P₃ 5-phosphatase. *b*, The distribution of marker enzymes for cytosol (inulin), Golgi membranes (galactosyltransferase, galtrans), plasma membranes (leucyl-β-naphthylamidase, LNA), endoplasmic reticulum (arylesterase, arylest), lysosomes (β-galactosidase, β-gal) and mitochondria (glutamate dehydrogenase, GDN). The results shown and the recoveries (see below) are for a single representative experiment. Data are plotted as the percentages of the total recovered activity or component found in each fraction, and values of <0.5% of recovered activity have been omitted from *b* for clarity. **Methods:** Hepatocytes, prepared and incubated as described previously²⁴, were homogenized using N₂ cavitation²⁵ in 0.25 M sucrose containing 3 mM TES (2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethane sulphonic acid), adjusted to pH 7.4 with KOH; 0.4 ml of a nuclei-free homogenate²⁵ was added to 0.4 ml of homogenization buffer that had been layered on the top of an isoosmotic density gradient of Percoll suspended in homogenization buffer (5.5 ml of density 1.052 g ml⁻¹ above 1.2 ml of density 1.074 g ml⁻¹). Gradients were centrifuged for 15 min at 73,000 g (mean) in a 10 × 10 ml angle rotor. Fractions (214 μl) were recovered, and their components and densities assayed as follows: Ins(1,4,5)P₃ 5-phosphatase (assayed as described in Table 1; recovery of loaded activity in fractions from gradient-85%); inulin (followed in a separate gradient in parallel, after addition of 1.5 μCi ml⁻¹ ³H-inulin to homogenate before centrifugation; 100% recovery); galactosyltransferase (ref. 24; 84% recovery); LNA (ref. 25; 96% recovery) (a large proportion of this enzyme activity distributes with glucagon-stimulated adenylate cyclase, an enzyme concentrated in the hormone-sensitive sinusoidal domain of the hepatocyte plasma membrane²⁵); arylesterase (ref. 24; 96% recovery); β-galactosidase (ref. 26; 111% recovery); GDN (ref. 24; 81% recovery).

Table 1 First-order rate constants for hydrolysis of inositol phosphates in liver preparations, corrected to the enzyme concentrations of the original tissue

Fraction	Ins(1,4,5)P ₃	First-order rate constant (s ⁻¹)			Ins(4)P
		Ins(1,4)P ₂	Ins(1)P		
Homogenate	0.148 ± 0.019	0.018 ± 0.022	0.007 ± 0.001		NA
Soluble	0.035 ± 0.005	0.028 ± 0.004	0.009 ± 0.002		0.0002
Particulate	0.095 ± 0.007	0.004 ± 0.001	0.003 ± 0.0004		NA
Washed particulate	0.065 ± 0.006	0.004 ± 0.0004	0.0009 ± 0.0003		NA

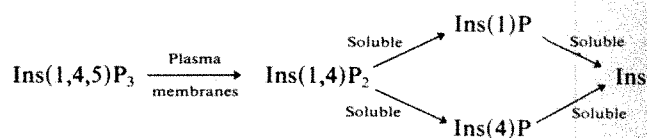
[4-³²P]Ins(1,4)P₂ and [4,5-³²P]Ins(1,4,5)P₃ were prepared as described previously^{15,17}. ³²P-Ins(1)P was produced by phosphodiesterase-catalysed hydrolysis of ³²P-phosphatidylinositol using a soluble liver enzyme preparation (details to be described elsewhere), and isolated by high-voltage electrophoresis²³. Rat livers were cleared of blood by perfusion of 0.25 M sucrose/5 mM Tris (pH 8.0) through the hepatic portal vein under pentobarbital anaesthesia. The livers were removed, homogenized in the same medium in a glass-Teflon Potter-Elvehjem homogenizer (20 strokes, 500 r.p.m.), and the homogenates filtered through a 300-μm mesh and made up to 3 ml per g tissue. Homogenates were centrifuged at 180,000g for 30 min and the supernatant and pellet made up to the volume of the original homogenate (to yield 'soluble' and 'particulate' fractions). Some of the 'particulate' fraction was again centrifuged and resuspended, to yield the 'washed particulate' fraction. The medium used for enzyme incubations comprised (in mM): 10 NaCl, 70 K⁺ glutamate, 30 KCl, 4 MgSO₄, 10 HEPES, 1 EGTA, pH 7.2. Ins(1,4,5)P₃ 5-phosphatase activity in this 'physiological' medium is rather more than half of that in the ionic strength medium used previously¹⁶, both for erythrocyte membranes and liver cell fractions. Incubations were in a final volume of 1 ml, containing 50 μl of the homogenate or cell fraction, and they were continued for an appropriate period (1 min to 4 h) in order to yield a first-order rate constant for the hydrolysis of the phosphate ester being tested. The substrate concentrations were in the ranges 40–300 nM (for Ins(1,4,5)P₃ and Ins(1,4)P₂) and 8–24 μM (for Ins(1)P). Incubations were stopped by adding 100 μl of 12 M perchloric acid containing 1 mM P_i, and released ³²P-P_i was estimated as described previously¹⁶. The values given are the means ± s.e.m. (n = 4). The approximate rate constant for hydrolysis of Ins(4)P is derived from the second exponential (that for ³²P-Ins(4)P) on a prolonged rate curve describing hydrolysis of [4-³²P]Ins(1,4)P₂. NA, not assayed.

On density gradient centrifugation of nuclei-free liver homogenates, the Ins(1,4,5)P₃ 5-phosphatase activity of the particulate fraction was largely associated with fractions of density 1.035 g ml⁻¹; these were rich in plasma membrane and Golgi markers, rather than those of mitochondria, endoplasmic reticulum or lysosomes (Fig. 1). Isolated nuclei showed little Ins(1,4,5)P₃ phosphatase activity (data not shown), and Prentki *et al.*¹¹ have also reported a low Ins(1,4,5)P₃ phosphatase activity in endoplasmic reticulum membranes from an insulinoma-derived cell line. Preliminary studies of highly enriched preparations of plasma membranes and Golgi membranes suggest that most of the Ins(1,4,5)P₃ 5-phosphatase is associated with plasma membranes, as in the erythrocyte¹⁶. The membrane-bound activity in liver was near-maximal over a fairly broad range of pH (~5.0–7.2), also like the erythrocyte enzyme. The erythrocyte Ins(1,4,5)P₃ 5-phosphatase is very sensitive to inhibition by Ag⁺ and Zn²⁺ (ref. 16), but problems of solubility prevented the use of these ions in the iso-ionic media employed in our experiments with liver cell fractions. However, we have recently shown that the erythrocyte enzyme is very sensitive to inhibition by Cd²⁺ (C. E. King, unpublished data), and the membrane-bound Ins(1,4,5)P₃ phosphatase of liver is similarly inhibited by this ion (Fig. 2). The membrane-bound Ins(1,4,5)P₃ 5-phosphatase activity was insensitive to concentrations of Li⁺ up to 10 mM and was inhibited only by high concentrations of F⁻ (k_i ~3 mM). The smaller proportion of the Ins(1,4,5)P₃ phosphatase activity that was present in the soluble fraction was studied in less detail (because of the problems inherent in assaying Ins(1,4,5)P₃ hydrolysis without interference from further hydrolysis of the liberated Ins(1,4)P₂), but its general characteristics appeared similar to those of the particulate activity.

The soluble activity that hydrolysed Ins(1,4)P₂ yielded both a labelled inositol monophosphate and labelled P_i. Because the Ins(1,4)P₂ used as substrate was labelled only in the 4-phosphate, initial attack must have occurred at either phosphate group. The labelled Ins(4)P produced during these incubations was slowly dephosphorylated. Ins(1,4)P₂ hydrolysis was much less sensitive to Cd²⁺ inhibition than was hydrolysis of Ins(1,4,5)P₃ (Fig. 2); it was, however, sensitive to inhibition by Li⁺ (k_i ~2.5 mM) and F⁻ (k_i ~0.2 mM). The soluble activity responsible for Ins(1)P hydrolysis, like the Ins(1)P phosphatase of brain¹⁷ and other tissues¹⁹, was sensitive to inhibition by Li⁺ (k_i ~4 mM) and F⁻ (k_i ~0.2 mM).

These results, obtained using incubation conditions similar to those within the cytosol, indicate that the pathways that inactivate Ins(1,4,5)P₃ and return its inositol to the free inositol

pool of the cell are as shown below:



These results have several interesting implications. First, hydrolysis of Ins(1,4,5)P₃ involves the selective removal of its 5-phosphate by a phosphatase which appears to be localized, at least in part, at the plasma membrane. This enzyme will effectively terminate the mobilization of Ca²⁺ by Ins(1,4,5)P₃, as it yields Ins(1,4)P₂ which is essentially incapable of mobilizing Ca²⁺ from intracellular stores^{7–12}. From the relatively limited results obtained so far, it seems that the Ins(1,4,5)P₃ 5-phosphatase of liver is very similar to that of the erythrocyte both in its plasma membrane localization and its inhibition characteristics. The function of the Ins(1,4,5)P₃ 5-phosphatase activity of erythrocyte plasma membranes is unknown, but it might be a remnant of a hormonal signalling system which functioned during the haematopoietic stages of the cells' development¹⁶. This would be analogous to the retention of the guanyl nucleotide-dependent coupling proteins of the adenylate cyclase system in mature erythrocytes (for example ref. 20). The similarity between the membrane-bound Ins(1,4,5)P₃ 5-phosphatases of liver and erythrocytes provides some support for the notion that the experimentally accessible erythrocyte enzyme may be useful for investigating the characteristics of the enzymatic mechanism(s) by which Ins(1,4,5)P₃ is inactivated in stimulated cells.

Hydrolysis of Ins(1,4)P₂ and of both isomers of InsP is much slower than hydrolysis of Ins(1,4,5)P₃, as also observed with blowfly salivary gland¹⁵. Thus it seems likely that under normal circumstances the former reactions control the rate at which inositol derived from Ins(1,4,5)P₃ is returned to the cellular pool of free inositol. In the presence of Li⁺, these reactions are substantially inhibited, and inositol is trapped in the phosphates^{19,21}.

The hydrolysis of Ins(1,4)P₂ by soluble phosphatase activity does not show a precise positional selectivity like that of Ins(1,4,5)P₃: the 1- and 4-phosphate groups are removed at approximately similar rates, and the released Ins(1)P and Ins(4)P are both more slowly dephosphorylated by soluble phosphatase(s). The studies reported here do not show whether a single enzyme, which is sensitive to inhibition by Li⁺ and F⁻, is responsible for hydrolysis of Ins(1,4)P₂ and of the inositol monophosphates, or whether a different enzyme is involved in

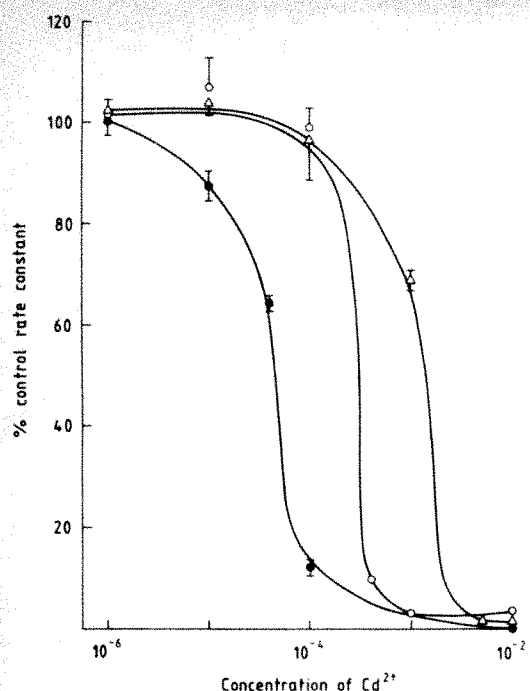


Fig. 2 A comparison of the sensitivities to inhibition by Cd^{2+} of particle-bound $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase (●), soluble $\text{Ins}(1,4)\text{P}_2$ phosphatase (○) and $\text{Ins}(1)\text{P}$ phosphatase (△) from rat liver. Enzyme activities were measured as described in Table 1, in the presence of various concentrations of Cd^{2+} . Results are the mean $\pm$ s.e.m. ($n \geq 4$). Values for s.e.m. $< 1\%$ have been omitted.

each reaction. Sherman and colleagues²¹ have recently identified small quantities of $\text{Ins}(4)\text{P}$ among the inositol phosphates that accumulate as a result of receptor activity in the brains of Li^+ -treated rats. It seems probable that this novel phosphate ester is produced from $\text{Ins}(1,4,5)\text{P}_3$, generated as a result of receptor activity, by the reactions shown in the scheme above.

An approximate extrapolation from the first-order rate constant given in Table 1 suggests that, in intact liver cells, the half life of $\text{Ins}(1,4,5)\text{P}_3$ is likely to be of the order of 4 s, suggesting considerably faster degradation of this compound than was reported in parotid fragments on termination of a stimulus^{13,14}. However, a recent report from Irvine and colleagues has shown that not only $\text{Ins}(1,4,5)\text{P}_3$, but also inositol 1,3,4-trisphosphate ($\text{Ins}(1,3,4)\text{P}_3$), accumulates in stimulated rat parotid fragments²², with the unexpected 1,3,4-isomer predominating. The reason only small quantities of $\text{Ins}(1,4,5)\text{P}_3$ were detected might be that this phosphate ester is degraded much more quickly than $\text{Ins}(1,3,4)\text{P}_3$; nothing is yet known about the pathway by which the latter compound is degraded.

We thank the MRC for project grant support and for a research studentship (to D.J.S.), and Eleanor Metcalf for her participation in the initial studies. We thank Alan Wallace for helpful advice on density gradient centrifugation.

Received 27 July; accepted 7 September 1984.

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A developmental gene product of *Bacillus subtilis* homologous to the sigma factor of *Escherichia coli*

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Sporulation of *Bacillus subtilis* involves sequential morphological and biochemical changes and is regulated by specific genes (*spo* genes) estimated to occupy more than 30 loci^{1,2}. A mutation in any one of these genes blocks the sporulation process at the corresponding developmental stage. Despite intensive genetic studies, the nature and function of the *spo* gene products remain unknown. Vegetative *B. subtilis* RNA polymerase core enzyme may interact with several sigma factors and discriminate among different classes of promoters³. During sporulation, new polypeptides are associated with the core enzyme which may have a central role in modifying its promoter recognition specificity^{3,4}. As a first step to understanding their function in the switch from vegetative to sporulation mode, several early sporulation genes have been cloned and analysed⁵⁻⁷. Here we report the cloning and nucleotide sequence of the *spoIIg* gene of *B. subtilis*^{8,9}. This gene encodes a polypeptide with a predicted relative molecular mass of 27,652 which contains a 65-amino acid region highly homologous to an internal part of the *Escherichia coli* sigma factor.

From a genomic library constructed as described previously¹⁰, but enriched in DNA restriction fragments of 2-6 kilobases (kb), we isolated a new recombinant plasmid that was able to transform to the *Spo*⁺ phenotype both *Rec*⁺ and *Rec*⁻ *spoIIg* recipients, indicating that the insert carried the entire *spoIIg* locus. The 3.6-kb insert was analysed by endonuclease restriction mapping (Fig. 1) and was further reduced by several subcloning steps. Ligation of a 1.1-kb *Pst*I fragment in the *Pst*I site of the pHV33 vector yielded plasmid pGSIIG11, still carrying the intact *spoIIg* locus, as shown by its transforming activity.

The 1,133-base pair (bp) nucleotide sequence of the pGSIIG11 insert (Fig. 2) was determined by the method of Maxam and Gilbert¹¹ according to the strategy outlined in Fig. 1. There is only one large open reading frame potentially encoding a polypeptide. As the requirements for translation initiation are more stringent in Gram-positive bacteria than in *E. coli*¹², a strong ribosome binding site is usually found in the vicinity of the translational start codon of *B. subtilis* genes. Such a sequence, AGAAGGGAG, appears at position 164 and precedes by six nucleotides an in-frame ATG codon. It seems probable that initiation of translation *in vivo* occurs at this codon, leading to the synthesis of a 239-amino acid polypeptide with a relative molecular mass (M_r) of 27,652. This polypeptide is rich in charged amino acids (33.5%) with an excess of basic residues over acidic ones (42 versus 38). These charged residues are not randomly distributed: the basic ones are clustered in the terminal

parts of the polypeptide (8 among the first 22 residues and 8 among the last 18 residues), while a stretch of 10 acidic residues is found in an internal region, between amino acids 135 and 163. These features may be significant for the biological activity of the *spoIIG* gene product.

The encoded protein seems to be synthesized in a very low amount as we have been unable to express it in *B. subtilis* maxicells in various experimental conditions; this suggests that the protein may have a regulatory role in sporulation. Note that the *spoIIG* gene is transcribed only in the early stages of sporulation and not during vegetative growth. This was demonstrated by hybridization experiments in which the gene was used to probe mRNAs isolated at different stages of growth and sporulation (results not shown).

To elucidate the nature of the polypeptide encoded by the *spoIIG* gene, we searched for homologies with known proteins stored in the Dayhoff protein sequence data bank (~2,300 sequences). An important homology was found with the product of the *E. coli rpoD*¹³ gene, the sigma factor. Figure 3a shows the alignments of the two related segments of the *B. subtilis spoIIG* gene product and of the *E. coli* sigma factor. These aligned sequences exhibit a remarkable similarity: of the 65 positions compared, 29 (44.6%) are occupied by identical amino acids. When chemically similar pairs of amino acids are taken into account¹⁴, this number rises to 44 (67.7%). It was not necessary to introduce gaps to maximize this homology. Furthermore, statistical tests^{15,16} show that the similarities between the aligned sequences are highly significant; the probability that such a similarity is fortuitous is $<10^{-9}$ (Fig. 3a).

Analysis of the corresponding nucleotide sequences also revealed a high degree of similarity at the DNA level (100 matches over 195 positions, that is 51.3%; see Fig. 3b). A striking feature of the similarities is their clustering into three blocks of 34, 35 and 30 bp, where 73.5%, 80% and 70% of the respective positions are occupied by identical nucleotides. As the codon usage is not the same in *E. coli* and in *B. subtilis* (as indicated, for example, by the respective usages of the *trpE* genes of these two organisms^{17,18}), such similarities strongly suggest that the two genes are evolutionarily related.

The regions shown in Fig. 3 correspond to different internal parts of the two proteins: residues 58–127 of the 239-amino acid polypeptide encoded by *spoIIG* and residues 375–439 of the *E. coli* sigma factor, a polypeptide of 613 amino acids¹³. However, their high degree of similarity strongly suggests a common function for these two protein domains. This leads to the attractive hypothesis that the *spoIIG* gene product may act as a specific sigma-like factor and that the region it has in common with the genuine *E. coli* sigma factor may similarly be involved in binding to the core RNA polymerase. This hypothesis is strengthened by the known functional and structural relationship between the *E. coli* and *B. subtilis* core RNA polymerases^{19–21}. Moreover, recent nucleotide sequence analysis of the *B. subtilis* σ^{55} structural gene (R. H. Doi, personal communication) indicates that the homologous region in the *E. coli* sigma factor and the *B. subtilis spoIIG* gene product is conserved in the major sigma factor of *B. subtilis*, σ^{55} , confirming previous immunological experiments²². One obvious possibility is that the *spoIIG* gene encodes σ^{29} , a polypeptide associated with RNA polymerase purified from *B. subtilis* sporulating cells and conferring on it new promoter recognition specificity²³. σ^{29} is a M_r 27,000–29,000 polypeptide^{4,23,24} and appears about 1 h after the onset of sporulation²³, the exact moment at which the temperature-sensitive period starts in strain 279.1, a strain mutated in the *spoIIG* gene⁸. However, sporulation involves the expression of multiple gene sets and could rely on the presence of several alternative sigma-like proteins. The *spoIIG* gene product could easily be one of these, as yet uncharacterized, transcriptional factors.

We thank Françoise de la Torre for technical assistance in cloning the *spoIIG* gene, Isabelle Giri for help with computer sequence analysis and Jocelyne Mauger for typing the manuscript. This work was supported by a CNRS grant (LA 136) to P.S. and J.B.

Note added in proof: The region of homology between the *B. subtilis spoIIG* gene product and the *E. coli* sigma factor has recently been found to be conserved also in the heat shock regulatory protein of *E. coli*²⁷.

Received 18 June; accepted 6 September 1984.

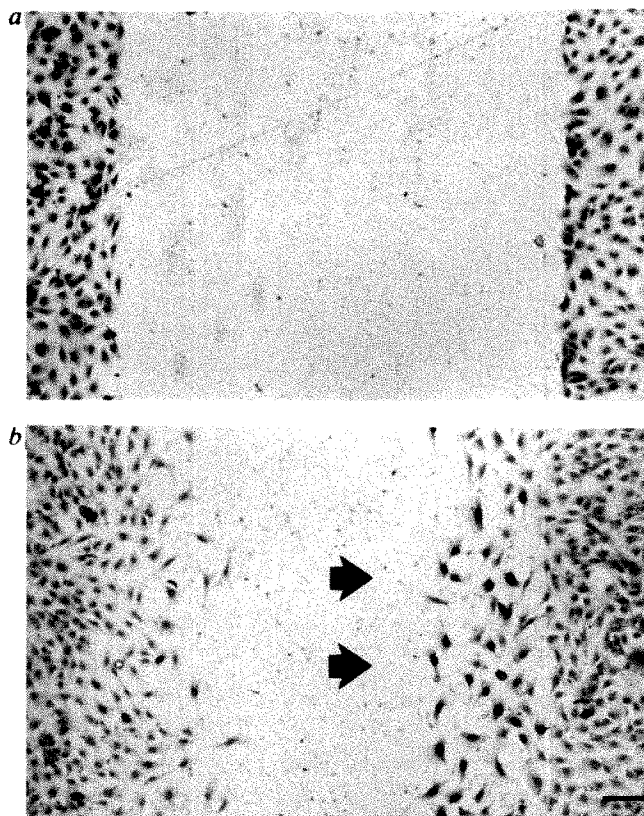
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Erratum

New evidence that growth in 3T3 cell cultures is a diffusion-limited process

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Nature **312**, 63–65 (1984)

BECAUSE of an editorial error, an incorrect figure was used in this letter. The correct Fig. 3 is shown below.



African marsupials— vicariance or dispersion?

UNTIL recently the marsupials were not recorded in Africa, but two recent discoveries in the Lower Eocene and in Lower Oligocene of North Africa have demonstrated the presence of this group of mammals on the continent. Their phylogeny and palaeobiogeography must, therefore, be reconstructed.

The discovery of marsupials in the Lower Oligocene of Africa has recently been interpreted as indicative of continuous land dispersion across the Tethys from Europe to Africa¹. Another recent discovery, however, proves that marsupials were present in northern Africa in the late Lower Eocene². The upper molar described from the north-west Sahara displays both primitive characters (the narrowness of the protocone) and very derived characters (dilambdodonty), which could suggest either immigration from a European centre of origin or survival of an ancestral stock common to South America and Africa before the opening of the South Atlantic (vicariance).

A vicariant origin for the African marsupials should not be excluded, because the time when the main marsupial dental characters appeared³, which would permit an evolutionary evaluation of African Oligocene and European late Eocene marsupials, is unknown. Unfortunately there is no way of testing the phyletic relationships between the Saharan and Egyptian forms, due to the scanty fossil record. On the other hand, European or Holarctic origin is supported by the continuous record of mammals of Holarctic affinities discovered in the Palaeocene⁴, Eocene^{5,6} and Oligocene⁶ of Africa, and by the absence of marsupials from the only known Palaeocene mammal fauna of Morocco⁴. The recent discovery of marsupials in the European Palaeocene⁷ reinforces this interpretation. However, several other palaeobiogeographic scenarios might also help to explain their presence in Africa. According to Sigé⁸, Africa could have been a land route between South America and North America, but there is no evidence of non-marsupial vertebrate dispersion to support this hypothesis.

Several authors have claimed that Africa could have been a link during early Cenozoic between South America and Europe for phorusrhacids⁹, mesosuchian ziphodont crocodiles¹⁰, characid fishes⁴ and marsupials⁷. As in the case of Africa, models which exclude Asia and the Indian plate need to be reconsidered. No true marsupial has been discovered in the Asian Mesozoic, but very few micromammals have been described from the rich mammalian assemblages of the Chinese early Tertiary. Additional information is provided by molecular data. For example, the structure and

sequence of neuropeptides¹¹ have recently shown that the most primitive living marsupials are Australian, thereby suggesting that Australia could have been the dispersion centre of the group. In conclusion, these new data demonstrate that the phylogeny and palaeobiogeography of marsupials is much more complicated than previously assumed, and should stimulate new research in many fields.

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BOWN AND SIMONS REPLY—Jaeger and Martin suggest a possible vicariant origin for newly discovered marsupials from the Lower Eocene of Algeria and the Oligocene of Egypt^{1,2}. These are the only metatherian remains ever recovered from the African continent, and the vicariance hypothesis suggests that these animals are survivors of an ancestral marsupial stock common to South America and Africa before the opening of the South Atlantic (before the early Cretaceous³). The distributions of at least the early Tertiary marsupials of Europe and North America might then have been derived from this remnant African stock, rather than having originated in Europe, as we have claimed² for the Egyptian marsupial, or in North America, as suggested by others^{4,5} for the Euramerican marsupials. Although a vicariance theory of origin of mammalian groups is in vogue, its application to this problem and to others is generally supported only by negative evidence, that is, the absence of a good fossil record. For African marsupials, a vicariant origin is the least parsimonious alternative and requires detailed speculation about the composition of a non-existent fossil record.

First, there are no known pre-late Cretaceous marsupials from South America (the late Cretaceous marsupials from Peru are virtually identical to North American late Cretaceous *Alphadon*⁶). Second, the only proposed marsupial of early Cretaceous antiquity from anywhere in the world is North American *Holo-*

*clemensia texana*⁷, a form that some feel is of doubtful metatherian affinity^{8–10}. Even if *Holoclemensia* is metatherian, vicariance biogeography would place the origin of the Metatheria in North America, in accordance with most current thought. Third, the combination of primitive and derived characters in the Eocene Algerian form is unknown in any other didelphid, including those from South America, and the Egyptian marsupial shares derived characters with late Eocene and early Oligocene marsupials from Europe. By a vicariant origin, the Didelphidae of South America and those of Euramerica would have to be polyphyletic, or else the South America didelphids were at some time reintroduced there from Africa via Euramerica. Fourth, Egyptian *Peratherium* is so like European species of the genus, that had the Old World didelphids originated in Africa and spread to Euramerica, the avenue of dispersal between Africa and Europe must have been irregularly maintained over many millions of years. The first three points are not supported by palaeontological evidence, and the last observation is directly opposed to what is known of Tethyan palaeogeography during the early Tertiary.

Although Jaeger and Martin are correct in observing that a vicariance origin for the African marsupials should be examined, there is no evidence to support their hypothesis involving the composition and dispersion of an unknown fauna. We believe that there is some evidence that the Egyptian marsupial was an early Tertiary immigrant from Europe. Marsupials were probably present in Europe during the Palaeocene¹¹, although they are as yet unknown from mammalian microfossils of that age in Africa. The Egyptian marsupial cannot have arrived in Egypt before the latest Eocene because of marine conditions there. Its appearance in the Egyptian continental Oligocene approximately coincides with the Tethyan regression, when Europe and Africa were presumably separated by less water distance than for many millions of years previously.

We also observe that the presence of a single stylar cusp on a narrow stylar shelf, in conjunction with the more or less dilambdodont molar structure in the specimen from the Eocene of Algeria, is so unusual for a marsupial as seriously to jeopardize its assignment to the Metatheria. The tooth appears to share at least as many derived characters with European *Remiculus* (Mixodectidae) and *Adapisoriculus* (Insectivora, *incertae sedis*) as it does with any marsupial.

Finally, we did not "... interpret the discovery of marsupials in the Lower Oligocene of Africa as indicative of continuous land dispersion across the Tethys from Europe to Africa", as Jaeger and Martin suggest. Rather, we observed that the palaeogeographical reconstructions do

not favour a direct land connection of Africa with Europe at that time².

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Cometary showers and unseen solar companions

THE existence of an unseen solar companion has occasionally been invoked to account for some unsolved astrophysical problem. The latest hypothesis, as proposed by Whitmire and Jackson¹ and by Davis *et al.*², is that such a companion, in a distant eccentric orbit, periodically passes through the Oort cometary cloud and causes a shower of comets from an also unseen inner Oort cloud reservoir. Impacts on the Earth from these repeated showers then results in periodic biological extinction events.

A shower of comets from the inner Oort cloud is expected to involve some 2×10^9 comets passing four times each within the orbit of the Earth, over a period of $\sim 10^6$ yr. This episodic flux is equivalent to the presently estimated steady-state cometary flux of 16 comets $\text{AU}^{-1} \text{yr}^{-1}$ (ref. 3) (brighter than $H_{10} = 11$) for a period of 500 Myr. Thus, the seven to nine cometary showers, spaced at 28-Myr intervals, for which a cratering record has allegedly been found⁴, would produce as many craters as the steady-state cometary flux over the Solar System's entire history.

Present estimates of the cratering rates on the Earth and Moon based on dated surfaces of very different ages⁵, are in agreement with one another, after allowing for differences in gravity-scaling and impact cross-section. Estimates of the expected cratering from Earth-crossing asteroids are roughly twice these values⁶; estimates of the cratering from comets range between 0.3 and 1.0 times the rate based on dated crater surfaces^{7,8}. Although it seems that the known Earth-crossing objects yield a total cratering rate on the Earth and Moon which is too high, the problem is not serious because of the large error bars in these calculations.

However, adding the flux predicted for cometary showers every 28 Myr makes the problem significantly worse. These showers result in an 18-fold increase in the mean cometary flux over the past 400 Myr, resulting in between 5.4 and 18 times as many additional craters on the Earth and Moon in that period, outside any reasonable error limits on these rates.

Even allowing this discrepancy, the probability that such a cometary shower would result in the impact of a 10-km diameter cometary nucleus on the Earth is about 0.055 per shower. Thus, major events like the Cretaceous-Tertiary (K-T) extinction might be expected on average every 510 Myr, considerably longer than is observed.

One can also consider the stability of the hypothesized 'death star' orbit. With a perihelion of 3×10^4 AU and a period of 28 Myr, the proposed companion star would have an aphelion of 1.54×10^5 AU. Monte Carlo simulation modelling of the dynamical evolution of a large sample of hypothetical stars starting with that orbit⁹ has shown that 23% escape the Solar System in 10 orbits or less, and 86% escape in $< 10^9$ yr. The average change in orbital period per orbit is 10%. Thus, it is unlikely that an unseen companion star could have remained in a constant period orbit, over the period suggested by the terrestrial extinction record, and impossible that it has been in that orbit since the origin of the Solar System.

If a more tightly bound original orbit were to be assumed, the problem of the increase in the terrestrial cratering record would only worsen because of the increased frequency of cometary showers implied. Also, the Oort cloud population would have been severely depleted by the repeated perturbations of the companion star, implying an immense initial cloud mass. If, on the other hand, one assumed that the companion star had been recently captured, an event with a probability of 10^{-13} , we would still expect an approximate doubling of the cometary cratering on the Earth in the past 300 Myr.

Finally, an additional problem with the death star scenario is that there do not seem to be many random events mixed with the periodic signal from cometary showers. Random Apollo asteroid impacts should produce a major extinction every 10^8 yr or so, and large (though not necessarily catastrophic) cometary impacts should occur with about one-third that frequency.

The proposed death star scenario, however interesting, has consequences which its proposers have failed to consider, and for which we have little evidence. If the periodicity in the fossil extinction record is indeed real, then some other mechanism must be found for creating it. It would seem wise to look for a period of which we are already aware, for example, the time between the Sun's galactic plane crossings is close to the required

value, though the extinction mechanism remains a mystery. Even so, this would seem a more worthwhile avenue for future study than continuing to chase an invisible star which does not exist.

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MULLER *ET AL.* REPLY—Weissman's objections are based, we believe, on a misunderstanding of the companion star model^{1,2} and a misinterpretation of the periodicity discovered in the crater data³. We will review the salient features of these before addressing Weissman's points directly.

In the companion star model, the Sun's companion has an orbit with a 26-Myr period, matching that seen in the mass extinctions⁴. During perihelion, it passes close enough to the Oort cloud of comets to precipitate a shower in the normally depleted 'loss cone' region swept clean by Jupiter and Saturn; this requires the companion's orbit to have an eccentricity greater than ~ 0.6 . The number of comets in the shower depends on the number in the inner part of the Oort cloud, but this number is not known within a factor of 10. If we take the nominal value of 10^{13} from the extrapolation of Hills⁵, we find the number of comets in a shower to be 2×10^9 and the number of impacts per shower to be 25; but these numbers could be as small as 4×10^8 and 5, respectively.

As stated in our original paper¹, and subsequently checked with detailed calculations⁶, the orbit we postulate is stable, with a half life of $\sim 10^9$ yr, not long enough for the companion star to have been in such a large orbit at the formation of the Solar System. As it is extremely unlikely that a star could have been captured by the Sun, the most likely scenario has the companion in a relatively tight orbit 5×10^3 yr ago, with perturbations from passing stars and molecular clouds over 5×10^9 yr expanding the orbit to the present one.

In Grieve's survey of impact craters on the Earth⁷, there are 58 craters with ages < 250 Myr, but only 29 of these have uncertainties in their ages of 20 Myr or less, and only 13 of these remain when we exclude the very recent (< 5 Myr old) craters; these appear in a table in our paper³. Our analysis of these craters showed a statistically significant periodicity, with period and phase identical

(within errors) to those found in the fossil record. On closer examination we found that most of the signal was coming from the 11 largest craters, those with a diameter >10 km. One would expect comets in a shower to be larger than the background comets if, for example, the comets from the inner cloud were more massive than those from the outer regions, as might be expected from an accretion model of comet formation. Of the 11 large craters, one is clearly not associated with the periodicity, and as many as two others may be chance coincidences. Thus, the evidence indicates that about 9 ± 3 of the 13 craters with precise ages originated from the periodic showers. (The uncertainty estimate includes both systematic and Poisson errors.) Stated another way, we conclude that $70 \pm 25\%$ of the craters were formed during showers.

Weissman claims that the total number of comets hitting the Earth from showers is substantially greater than the number hitting in the time between showers—this is not necessarily true in our model, and the impact data indicate that the numbers may be roughly equal. Perhaps Weissman's confusion comes from a poorly worded statement in our original paper¹ that the number of comets that arrive in a single shower "may be as much as one or two orders of magnitude greater than the number that arrive between showers". In this statement we were referring to the effects of the companion star alone, and were not including comets whose infall is triggered by random passing stars. Weissman states near the end of his letter: "there do not appear to be many random events mixed with the periodic signal"; as stated above, we believe that $30 \pm 25\%$ of the impact craters (principally those with diameters <10 km) may be random in arrival times.

Weissman says that the probability of a large comet (with a 10 km nucleus) hitting is small, and that such impacts are likely to occur every 500 Myr, a period "considerably longer than is observed". Only one such large impact has been proven in the last 250 Myr—the one at the end of the Cretaceous. The crater for this impact, if it still exists, should be 100–200 km across. (If the crater is on the sea floor it would not have been found.) All the other extinctions during this period were smaller, with associated craters 10–100 km across. Weissman claims there should be one large impact in 500 Myr; the data show one in 250 Myr. Although we do not necessarily accept Weissman's number (he does not give a derivation) we find no significant discrepancy with the data.

If the orbit of the companion star were once much closer to the Sun, as is necessary in our model, then Weissman objects that the showers would have been more intense in the past. This is true, but is not an objection. In fact, there is clear evidence that bombardment in the remote past was once much greater than in the

last 250 Myr. The 'late heavy bombardment' of the Moon (when most of the lunar craters were formed) ended 3.9×10^9 yr ago, conceivably when the companion star was scattered to a larger orbit. Weissman correctly states that an ancient close-in orbit would have severely depleted the Oort comet cloud, implying that there was originally an 'immense cloud mass'. As the mass of this early cloud could still have been much less than the mass of the companion star, we can see no valid objection. If the companion star exists, then we certainly will have to change our models of the dynamics of the early Solar System. But inconsistency with the models is not the same as inconsistency with the data on which those models were based. We know of no data inconsistent with our model of a solar companion star, and we know of no alternative model consistent with the measured period and phase of the impacts.

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WHITMIRE AND JACKSON REPLY—The contribution of shower comets to the mean cratering rate need only be comparable to the (assumed) steady-state random contribution to produce a strong signal in the Fourier spectrum of dated craters. The number of terrestrial impacts resulting from a shower of 2×10^9 comets, brighter than $H_{10} = 11$ and with perihelia ≤ 1 AU, is given by the product of (2×10^9 comets) and (4 perihelia passages per comet) and (2×10^{-9} impacts per perihelion passage), which is equal to 16 impacts. According to Weissman¹, a long period comet, brighter than $H_{10} = 11$, has a mass $\geq 5 \times 10^{14}$ g and produces a crater ≥ 20 km in diameter. The mean cratering rate, for craters ≥ 20 km, over the last 300 Myr is² $0.35 (\pm 0.13) \times 10^{-14} \text{ km}^{-2} \text{ yr}^{-1}$, which corresponds to ~ 50 impacts of comets, brighter than $H_{10} = 11$, per 28 Myr. Thus the shower contribution to the mean cratering rate is less than the total rate and comparable to the mean background rate.

According to Wetherill and Shoemaker³, a random 10-km asteroid is

expected to impact on the Earth about every 50 Myr. The absence of any random extinction events⁴, and the evidence that major extinctions occur over intervals of ~ 1 Myr, both suggest that the primary extinction mechanism may be associated with the enhanced impact rate (≥ 30 times background) during a shower, rather than with a single catastrophic impact, although such an event should also occur occasionally.

The stability of the companion's present orbit towards stellar perturbations and the galactic tides has recently been investigated by Hut⁵ and Hills⁶. These results indicate that the orbital period should random-walk away by 10–20% over a 250-Myr interval, a result not obviously incompatible with the cratering and extinction data. The evolution of the companion from a tighter orbit⁷, and the associated cratering rates, remain to be investigated in detail.

Sepkoski and Raup⁴ have recently extended their original analysis and have further restricted the uncertainty in the extinction period. They find the period to be 26.2 ± 1 Myr and conclude that this is incompatible with the 33 ± 3 -Myr interval between the Sun's galactic plane crossings.

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The maximum entropy method for data analysis

IN data analysis it is very satisfying when a useful technique, which has arisen in a more or less *ad hoc* manner, finds respectability as a manifestation of some formal theory. Skilling¹ invokes this argument for using the maximum entropy method for data analysis. Experience that the procedure often seems to work quite well is strengthened by reference to recent theoretical work. Skilling's conclusion is that maximum entropy is the only regularization method that should be used for a very wide range of data analysis. I suggest that this claim is unjustified on both pragmatic and theoretical grounds.

Let us confine our attention to one specific application, the enhancement of images as illustrated in Fig. 1 of Skilling's paper. What seems to me the major advantage of the maximum entropy method over many, but not all, other regularization

procedures, is the practical one that the resulting image, in being non-negative, automatically represents a valid solution. This results from the presence of $\log p_i$ in the formula for entropy. With other procedures, the values obtained for some p_i might be unrealistically negative. Even then, however, the complete solution could well be quite adequate in many applications and could well be attained with comparatively little computation.

In most applications of regularization, the regularization functional, exemplified in this method by entropy, S , incorporates some prior belief about the local smoothness of the true image. For example, suppose the pixels are ordered so that p_i and p_j are likely to be similar if i and j are close together; then one might consider, as an alternative to S , a criterion such as $-\sum(\log p_i - \log p_{i+1})^2$. At a higher level, one will know, *a priori*, that Fig. 1 is a smudged photograph of a car. The entropy functional does not permit representation of spatial ideas of this type. Certainly the cross entropy $S = -\sum p_i \log(p_i/m_i)$ allows for some acknowledgement of a prior model m but leaves the question of how to obtain m in any given application. There is the *ad hoc* suggestion of using the data to produce a preliminary m , but this would require using the data twice, first to find m and then to carry out the regularization. This is pragmatically reasonable, if not inevitable, but cannot be regarded as being formally respectable.

My principal point, however, is to dispute the main thrust of Skilling's article, that the maximum entropy method for data analysis, as used in the production of Fig. 1, stands in splendid isolation, on fundamental grounds. This statement is based on the axiomatic work of Shore and Johnson² and of Tikochinsky *et al.*³. They show that the choice of a probability distribution required to fit certain linear constraints has to be made on the basis of maximum entropy if certain axioms have to be satisfied. Typically, the effective constraints in the image-processing problems are highly nonlinear, and are expressed by the intensities themselves, not their proportions. It is not obvious that the mathematics of Shore and Johnson carry over to such a problem, and I cannot accept that their axioms, particularly those of subclass and system independence, are relevant to the regularization context. Tikochinsky *et al.*, in their discussion of reproducible experiments, specifically require the p_i to be probabilities; the assumption of linear constraints is vital, and even their definition of reproducible experiments does not correspond with the interpretation given by Skilling.

Thus, the maximum entropy method for data analysis is a useful tool, with some practical advantages and disadvantages over other regularization procedures. It is mildly interesting that there are links with the formal work on the principle of maximum entropy but the parallel simply

has not been shown to be exact enough to disallow flexibility in choosing a procedure to clarify Fig. 1 of ref. 1. As 'consistency', as defined by Shore and Johnson, seems irrelevant in this context, we need not fear the charge of inconsistency by rejecting entropy.

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SKILLING REPLIES—I find the arguments for using maximum entropy to be deeply compelling. In fact, I am unable to improve on Titterington's own words: "the maximum entropy method ... stands in splendid isolation, on fundamental grounds". Quite so. However, he takes me to task for not going beyond the description of an image as a set of probabilities/proportions to incorporate "some prior belief about the local smoothness of the true image". In this he has raised a most important point: clearly, the simple entropy formula $S = -\sum_i p_i \log(p_i/m_i)$ does not include any such belief; just as clearly, maximum entropy is losing power by ignoring this, provided of course that it is realistic to expect smoothness, or spikiness, or cars, or whatever in one's images.

I hold that these more complicated situations should ideally be handled by extending a technique which is basically correct rather than by invoking *ad hoc* alternatives. As Titterington's preference for smoothness tends to zero, I would like his formulae to reduce to the simple entropy form which is the compelling solution to that problem. This is because the regularization function, which expresses my preference for different shapes of image, should be independent of the particular form of the data to be measured. Logically, my preferences precede the data, so that I should use the same function whether I have convolution data (as in the car example) or marginal data (as in the theoretical discussion).

Fortunately, maximum entropy can include such preferences in an easy and natural way. The trick is to develop the identification of p with the image. The simplest identification is to let i be a single index ranging over the cells of the image, and p_i the corresponding proportion of flux. However, we can also let i be a composite index ranging over pairs of cells, and p_i the corresponding product of fluxes (itself a proportion). The model m_i can now incorporate pair correlations between cells as well as simple position-dependent information. If we take i to be a highly composite index, the model can encode correspondingly subtle details of prior knowledge. The arguments for using maximum entropy still hold, and we have been exploring this development. True, I

do not (yet?) know how to encode 'smoothness' in an absolute as opposed to *ad hoc* fashion. Nevertheless, I hope I have answered constructively Titterington's first major comment.

Concerning the relevance of Shore and Johnson's axioms¹, I do not think that the distinction between linear and nonlinear constraints is important here. The form of the experimental constraints is quite separate from the form of one's prior belief about the observed image, as coded in the regularization formula. Shore and Johnson explicitly state that their mathematics include nonlinear constraints. They use the technical trick of replacing a (convex) nonlinear constraint by that set of linear inequality constraints which defines the hull of the nonlinear constraint. Tikochinsky *et al.*² do not state this, having had physical applications in mind, but clearly the same trick can be used.

If the experimental constraints include dimensional information on intensities, the maximum entropy image will be accompanied by a dimensional number describing its normalization. Defining the regularization in terms of proportions rather than intensities merely requires the resulting image to have the same shape whether the data refer to microwatts or megawatts. The point about the subclass and system independence axioms is not that they hold for an arbitrary collection of data (such as a blurred photograph), but that they are compelling for particular collections (such as marginals). If one particular type of data forces me to use entropy, then I will consistently use entropy for other types also.

I admit that the use of entropy in data analysis imposes an interpretative gloss on the Shore and Johnson¹ and Tikochinsky *et al.*² papers, but I was writing a News and Views article, and I believe that these authors themselves approve of the application. We have to do something in data analysis: I am prepared to use probabilistic results to help to find sets of proportions. Probabilities—not that we need them anyway—and proportions are isomorphic: probabilities are just proportions in a sample space, and to each set of proportions there corresponds a probability distribution. Finally, I return to Titterington's remarks defending flexibility of choice. Maximum entropy using the composite (multi-cell) identification allows great flexibility in choosing the analytical procedure, but it also gives a framework within which choices can be related quantitatively to specific types of prior knowledge and discussed in a logical as opposed to a pragmatic fashion.

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Past probabilities

Patrick Rabbitt

Genius, Creativity & Leadership: Historiometric Inquiries.

By Dean Keith Simonton.

Harvard University Press: 1984. Pp.231. \$22, £17.60.

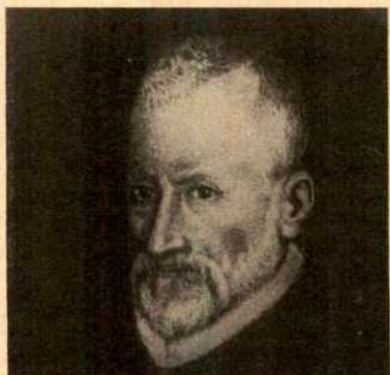
DEAN Simonton is a prescient man. While the rest of us glumly accept that our specialisms are of little interest to colleagues trapped in their own obsessions, he has developed a career as a "historiometrician". In any Common Room in the world, after the initial difficulty of explaining what this means, he can emerge as an expert on the Great Academic Post — Prandial Topics of All Time. Do higher degrees actually hinder creativity? Can we quantify charisma? Is history, especially the history of science, shaped by individuals or by Zeitgeists? Does the physical presence of a commander really affect the outcome of a battle? Are important scientific discoveries typically made independently by several people, or by isolated individuals? And much, much more.

Simonton offers more than dinner-table expertise. He is widely read, and has training in statistical methods the principles of which he tries to transmit in five appendices. Perhaps these will not help people without, or impress people with, a similar background, but he undertakes to bring recent analytical ideas to his material. This is interesting, since the *post hoc* detection of statistical associations in sub-sets from vast bodies of potential data can easily persuade us of very strange things.

Does Simonton's use of some up-to-date statistical techniques reduce uncertainties about assumptions of causal relationships in very large, uncontrollable, *post hoc* data samples? Well, not as such. When the logic behind selection of numbers for analysis is dubious, elaborate statistical methods cannot help. For example, on the whole Simonton favours the view that higher education stunts intellectual flexibility. He re-analyses Cox's (1926) collection of biographical details on "301 geniuses" extant between 1450 and 1900. Very few of them took higher degrees — which, therefore, are at least uncorrelated with originality? The conclusion may well be correct, but Simonton's argument requires some odd assumptions.

One is that the content, and the social and career implications of "higher degrees" have not changed since 1450. Simonton apparently reassures himself by computing that the incidence of higher degrees within Cox's sample did not change between 1450 and 1900. This is interesting, but hardly germane. We can only discover whether incidence of higher degrees in the Cox sample is atypical by comparing it against incidence in the reference popula-

tions from which it was drawn. To do this we must take into account enormous population growth since 1450, the consequent, likely, changes in the *percentages* of the population who could achieve historical recognition of pre-eminence in successive centuries, and the changes in the percen-



Royal College of Music



Royal College of Music

Composing originals? Palestrina (top) and Schoenberg.

tages of the population examined who ever experienced higher (let alone "higher higher") education from 1450 to 1900. Even if such a tedious computation were attempted, the Cox sample of 301 spread over four-and-a-half rapidly changing centuries would certainly be too small to allow any definite conclusion. It is not that historiographers do their sums wrong, it is that apparently they would rather analyse inadequate data than hold their peace.

Historical evidence will always be patchy. This makes it important for historiographers to recognize when they do not have enough data to answer a question. Simonton is intrigued (pp. 165–166) by Stewart's analysis that the familial birth order of "British Prime Ministers" (how many examined?) and Presidents of the United States (38 studied) appears to correlate with the types of political situation faced by their cabinets. Elsewhere he convinces himself that birth order is a potent force in determining personal style, and

feels this shows that events call forth the necessary person. Perhaps, but hold on. We have at least five birth-orders (only child, eldest, youngest of two, middle of three or more and youngest of three or more) and if we take only the four political scenarios distinguished by Stewart (civil conflict; international crisis and warfare; peace; and revolution), to show that one of these variables (birth-order) predicts even 10% of the variance in assignment to the other (political scenario) would require data from a far larger sample than Stewart provides. There are, indeed, well-established associations between birth order and intelligence test scores (5% of variance or less) but samples of thousands have been necessary to detect them.

The book is shot through with intriguing examples. For instance, can we find out whether originality in musical composition is a function of historical period? Simonton considered the first six notes of 15,618 themes produced by 479 "classical" composers between 1500 (Josquin de Pres) and 1950 (Schoenberg and Shostakovich). He examined the first-order transitional probabilities between notes in each of these note strings and related them to a (here undefined) baseline computation for all transitional probabilities between notes. Note strings were classified as less original if their transition probabilities coincided with, and more original if they deviated from, the frequency of two-note transitional probabilities in the (undefined) source transition matrix. On this basis Simonton plots his composer-originality by year from 1500 to 1950 and finds, overall, a steady increase in thematic originality over time, with sinusoidal variations peaking at about 1650 (Palestrina) and 1920 (Schoenberg), and with a severe trough between 1750 and 1820 (Haydn, Mozart, Beethoven and Schubert).

I leave others to argue how far two-note transitional-probabilities can be a valid index of thematic originality because I am entranced by the possibility of a splendid computational tautology: if the splendour transition probability matrix is, as one must suspect, computed from *the entire corpus of 15,618 themes collected over 450 years*, this would mean that the earlier a composer flourished the more chance his work had to influence his successors and so the greater was his likely *indirect* influence on the baseline against which he was judged. The steady increase in "originality" over 450 years is at once explained. Further, the more eminent a composer the more he would influence his contemporaries and successors, and so the more substantial would be his indirect contribution to the "baseline" matrix. Hence the historical plunge from "originality" shown by Mozart *et al.*, who are very like the matrix which their influence determined. If we leave out of consideration such perturbing factors as the increased diffusion of musical ideas between geo-

graphically remote cultures as communications improved over the centuries, it may be that we can extract one conclusion from Simonton's elaborate computation: those whose works have survived longest, and those whose works are most often performed, shape the "norms" in musical composition!

Sophisticated mathematical models may be misleading in another way — they may provide *precise descriptions of numerical data* which are quite implausible in terms of real-world *processes*. Simonton elegantly reviews data on changes in human creativity in politics, arts and sciences between the ages of 20 and 70. Alas, though cheerful exceptions abound, the unmistakable trend is for peaks to be reached between 35 and 45 with an accelerated decline thereafter. He neatly fits an algebraic model to the data (pp.110 and 111), and on p.109 describes the functional implications of this model:

Each creator uses up [*sic*] this supply of creative potential [determined individually — perhaps at birth?] during his or her productive career by transforming potential into contributions.

I wish that I could believe that, by prudent inertia, I may yet reserve some "creative potential", undissipated by book reviews and unclouded by time, to astound my clapped-out contemporaries when I reach my 80s.

Incidentally it is encouraging for common sense to note that Simonton's algebraic statement of his model does not actually have this, or any other, clear functional implication. It seems to be a neutral equation for fitting empirical data using one invariant constant (*e*), two other constants empirically derived for particular disciplines (*a* and *b*) and a fourth constant empirically derived for individuals (*c*). It is not a parsimonious equation.

Simonton is clearly a very hard-working academic, and seems sincerely anxious that his subject should help us to understand ourselves better, to learn to anticipate and to avoid political violence, and to give each other just credit for real achievements. His book will be marvellous bed-time reading for any academic with a curiosity about historical or statistical matters. Only part of its entertainment value lies in the detection of charming errors and non sequiturs. Simonton has an acute sense of *really* interesting, though possibly insoluble, problems, and whenever he seems to go wrong he sets us the important task of wondering whether, and precisely how, something useful may yet be said. In spite (surely?) of the most extreme temptations he brings to his material no detectable humour. But, for the reader with his own sense of fun, this will be a delightful book. □

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Cue for cooperation

F.H. Bronson

Mammalian Semiochemistry: The Investigation of Chemical Signals Between Mammals.

By Eric S. Albone.

Wiley: 1984. Pp. 360. £29.50, \$57.

MOST mammals are nocturnal and by human standards they are quite small. Thus they rely heavily upon their chemical senses when interacting with other members of their own species, avoiding predators or searching for food. Chemical cues play many crucial roles even in mammals that are larger, more diurnal, and hence more visually-orientated. The term "semiochemical" encompasses all such cues, whether they originate from plants or animals, and incorporates the concept of communication via pheromones.

By its very nature, the broad study of chemical cueing requires close collaboration between the chemist and the biologist; as witnessed by its impact on entomology, the result can be immensely rewarding. Research on mammals, however, has thus far been dominated by behaviourists and physiologists interested strictly in communication between members of the same species. Dr Albone is one of the few chemists whose primary interest is in mammalian chemical cueing, and this is the first account of the subject written by a chemist. The strengths and weaknesses of the book are those that might be expected when a scientist of one discipline looks at a complex subject that has its roots in another, markedly different discipline.

The first two chapters of the book introduce the subject and note the potential contributions and limitations of chemical investigations; of particular interest here is the author's emphasis on the

probable complexity of most mammalian chemical cues. The next six chapters deal with the potential sources of the cues and the types of compounds that may arise from these sources. Thus the skin, scent glands, urine, breath and the reproductive tract are considered in detail. Unfortunately much of this discussion relies upon data collected in human beings, and in only a few cases are sources and compounds related directly to functions (this, it should be said, is not the fault of the author, but an accurate reflection of the state of our knowledge). In a further chapter that should be read by everyone interested in the topic, Dr Albone considers the important role played by micro-organisms in shaping odours. Finally, a guest contributor, Dr Stephen Shirley, has written a nice primer on the neurophysiology of the chemical senses.

While this is a historically important book, dealing as it does with a young and rapidly developing area, it will probably fail in its stated objective of bringing together biologists and chemists. Much of what the biologist finds exciting about the subject is treated only superficially — the integration of chemical cues with information provided by the mammal's other senses; the shaping of responses by experience and context; the adaptive functions of signalling systems; and so forth. On the other hand, one can hope that the book will attract more chemists into semiochemical research, and all biologists interested in chemical cueing certainly should read it because it will help them understand how a chemist approaches many of the problems on which they work. Ultimately, real progress will occur only through cooperation, and scientists in both disciplines need to understand much better how their opposite numbers view this challenging field. □

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SOM introduction

P.W. Hawkes

Theory and Practice of Scanning Optical Microscopy.

By Tony Wilson and Colin Sheppard.

Academic: 1984. Pp.213. £25, \$39.50.

THERE are not many types of radiation for which high-quality, two-dimensional, intensity-sensitive recording media exist. Light and electrons are exceptional in this respect, so that (apart from a few early scanning electron instruments) scanning microscopes were developed long after their fixed-beam counterparts had become commonplace. It is, however, gradually being recognized that the optics of scanning systems and the sequential nature of the image-forming process possess

attractive features in their own right, with the result that commercial scanning electron microscopes have been with us for nearly 20 years and commercial scanning transmission electron microscopes for about a decade. Now, as this book convincingly demonstrates, the scanning optical microscope (SOM) is beginning to supplement its conventional ancestor.

No two people are better qualified to write a book on SOMs than Tony Wilson and Colin Sheppard, for almost all the work on the theory and performance of these instruments has come from their Department of Engineering Science in Oxford. In extremely simple language, and using mathematics that anyone familiar with Sections 9.5–10.5 of Born and Wolf's *Principles of Optics* will find easy to follow, they take us through the theory of image formation in the SOM. The two operating modes — essentially large

detector and small detector — are explored in great detail, though if I have a criticism of the book, it is here: the authors are so familiar with the difference between these modes (Type 1 and Type 2 or confocal microscopy) that they have not, I think, realized that this distinction may not seem so important to newcomers.

That apart, the book covers all of the principal aspects of scanning optical microscopy: the many detector modes and special operating conditions, depth discrimination and depth of field, ultra-high resolution, practical details of the optical elements of the SOM, applications to semi-conductors and some newer non-linear techniques. There is also a chapter on a scanning microscope which, ironically, mimics the fixed-beam instrument by furnishing the signals from the individual pixels almost simultaneously but which re-

tains the attractive features of the confocal arrangement.

This is the first book to be devoted entirely to the SOM and it is remarkably successful. A vast amount of material is presented very readably within its 200 or so pages, and practising microscopists — many of whom do not yet know what scanning, particularly scanning combined with electronic or computer image enhancement, has to offer them — will find it very helpful. The analysis of image formation will also be of some interest to students of the STEM, though the analogy between the two families of instruments is richer and more complex than is indicated here. □

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Flying tonight

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Bats: A Natural History.

By John E. Hill and James D. Smith.
British Museum (Natural History)/University of Texas Press: 1984. Pp. 243. £15, \$24.95.

The Lives of Bats.

By Wilfried Schober.
Croom Helm/Arco: 1984. Pp. 200. £13.95, \$24.95.

The past decade has seen a burgeoning of research interest in the second largest order of mammals, the Chiroptera, or bats. Such research is now serviced by international conferences and symposia, as well as by several newsletters, one of which has recently evolved into a dedicated journal. There is a certain sense of urgency about current work, to discover more about these mammals as outstanding examples of evolutionary development and adaptation before they decline in numbers even further. Bats are under pressure throughout the world — the use of agricultural insecticides, loss of natural roosts in caves and deciduous trees, and more recently the increasing application of remedial timber treatments in houses used as roosts have all contributed to a drastic fall in populations.

Accompanying recent activity among scientists has been a similar rise in public enthusiasm for bats. So it is appropriate that two general books on the subject should now appear at the same time.

Hill and Smith's *Bats: A Natural History* contains a wealth of up-to-date information on the world's bats in a well-written and easily assimilated form. The publisher's blurb commends the book to both the amateur naturalist and the serious student of zoology. While the treatment somewhat varies in depth, my own feeling is that it will be particularly useful as an introductory account for professional

biologists as well as for students.

The book goes well beyond the natural history approach implied by the title, as the authors have taken every opportunity to include physiological information in their reviews of thermoregulation, reproduction, echolocation and flight. Surveys of food habits and feeding, and population ecology, are also included, together with a systematic treatment of all bats at the family level. Hill and Smith are both systematists, and in their essay on the origin and evolution of bats they put forward the controversial theory that the Megachiroptera and Microchiroptera are not closely related, and that wings and flight have developed independently in these two groups of flying mammals. A chapter on man and bats contains a fascinating section on folklore, superstitions and legends, and includes a great deal of hitherto unpublished material. By contrast the authors are at their least engaging when writing about form and function.

The text is illustrated with excellent line diagrams, although several of the photomontages are of poor quality and are not closely related to the text. But overall this is a very useful book, even if it lacks that unbridled enthusiasm for all aspects of bat biology and a concern about their plight that is so evident in Wilfried Schober's *The Lives of Bats*.

Schober is a neurobiologist who has studied bats all his professional life. His account of their general biology is as authoritative as it is readable, and is ideally suited to the increasing number of amateur bat enthusiasts looking for an introduction to the subject. It has been sympathetically translated from the original German by Sylvia Furness, and revised by Tony Hutson, and is well illustrated with colour and half-tone plates which Schober has collected from colleagues around the world. □

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Molecular orbitals: into the age of computer graphics

Jeremy K. Burdett

A Pictorial Approach to Molecular Structure and Reactivity.

By Robert F. Hout Jr, William J. Pietro and Warren J. Hehre.
Wiley: 1984. Pp.404. £46.20, \$99.95.

W.L. Jorgensen and L. Salem's classic work, *The Organic Chemist's Book of Orbitals*, was published by Academic Press in 1973. It is a mark of changing times that whereas that volume included only rather crude, wire-frame drawings of orbitals, the present one contains glossy, computer-generated, Daliesque visions of the molecular orbitals of simple systems.

Indeed this is quite an unusual book altogether. It is largely composed of photographs of orbitals, with a couple of dozen pages of supporting text. For those of us who construct molecular orbital diagrams by doodling on the back of envelopes, it is pleasing to see how the shapes of the orbitals we sketch really look, and that *ab initio* computations confirm the general results that we get from simple ideas. Some of the photographs in this enormous undertaking are somewhat murky, while others are very complicated and resemble a piece of small intestine. But overall the pictures are remarkably effective in depicting the shapes of orbitals as they really are. Surely the age of computer graphics has arrived!

For the chemist who has an understanding of how the level structures of these molecules arise, some interesting points arise in the book. The differences in the shapes of the orbitals of the isoelectronic sequence CH_3 , NH_3 , OH^+ and PH_3 , for example, are really very striking. The spectrum of chemical behaviour shown by these species is much easier to understand with the assistance of these pictures than with the information provided by a conventional energy-level diagram.

I have one main criticism of the book. Although there are indeed nuggets of wisdom in the text supporting the pictures, there is just not enough in the way of chemical comment. For example the orbitals of ferrocene (the *bête noire* of *ab initio* theorists) command six pages of pictures and a measly four lines of text. There is much more to be said of the chemistry associated with the pictures than the authors give us here. Although in this sense the book does not live up to its title, it is by no means an unworthy successor to Salem and Jorgensen. □

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Developing theories of evolution

J.S. Jones

Mechanisms of Morphological Evolution: A Combined Genetic, Developmental and Ecological Approach.

By Wallace Arthur.

Wiley: 1984. Pp.275. £19, \$36.

ANCIENT philosophy had a convincing explanation for earthquakes: they result from uneasy movements by the tortoise upon which the globe rests. Biologists have long been satisfied by similar explanations of the enigmatic in terms of the mysterious. In the study of evolution such theories include the role of the devil in placing fossils in rocks, of "hopeful monsters" in the origin of species, and — most recently — of mutations in the genes which are supposed to control development in producing the transitions between major taxonomic groups.

The preface to Wallace Arthur's book tells us, once again, that "current evolutionary theory in terms of neo-Darwinism is inadequate as an explanation of long term evolution". The remaining 275 pages largely fail to provide a convincing alternative. Unlike many post-Darwinians, Arthur accepts that modern evolutionary theory is not necessarily wrong simply because it is widely accepted. Indeed, parts of his own theory have a refreshing conventional as, in the model put forward here, natural selection plays a major role in large scale evolution. Selection is said to act on the "D-genes" which are arranged as a "morphogenetic tree" to control development. "N-selection" depends on competition among members of a species while "W-selection" acts on the course of gene frequency change in expanding populations.

The book is an ambitious attempt to synthesize information from ecology, developmental biology and genetics. It is relatively free of what was known to an earlier and perhaps more cynical generation of evolutionists as "Wadswallow" — the posy of analogies (epigenetic landscapes, the relation between genotype and phenotype seen as a circus tent) with which even as distinguished an embryologist as C.H. Waddington was forced to disguise our naked ignorance of the genetic basis of body form. However, it remains the case that the theory developed here rests heavily on a class of genes whose very nature is speculative, and on modes of selection which have never been demonstrated to occur in natural populations.

One of the strengths of the book lies in

the author's ability to draw on the literature of developmental biology as much as on that of evolution. But although the references cited include a number from 1983, it is only in the past few months that information has begun to appear which suggests that there is indeed an evolutionary relationship between the genes which control development in different animals. We now know that the genetics of early development of insects with body forms as different as grasshoppers and flies is extremely similar, and that there has been little change in some of the genes which underlie differentiation in organisms separated by many millions of years of evolution. For example, the homeo box is a sequence which is closely related to mutations causing developmental abnormalities in *Drosophila*. It is found also in *Xenopus*,

chickens and human beings, suggesting that there is a fundamental similarity in the processes of pattern formation which has been conserved in the face of great changes in form during evolution. These genes regulating development therefore appear to be more — rather than less — resistant to the accumulation of mutations than are many of those which code for structural proteins; an observation which contrasts with the prediction of theories which emphasize the importance of developmental mutants in the origin of major groups. In a few years we may find that Darwinism has taught us more about the genetics of development than developmental biology can tell us about the processes of evolution. □

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In the soup

A. E. Walsby

The Ecology of Freshwater Phytoplankton.

By C. S. Reynolds.

Cambridge University Press: 1984.

Pp.384. Hbk £30, \$54.50; pbk £12.50, \$24.95.

EACH springtime in lakes of temperate climes brings the stirring of a thin alphabet soup of phytoplankton: *Asterionella*, the *Biddulphiales*, *Closterium*, *Dinobryon* and the *Euglenales* . . . The soup thickens as the temperature rises and the days lengthen; species *a* outcompetes *b* to form the spring bloom this year; species *c* which won the race last year when the weather was colder, has not shown itself at all; and noxious *d*, which dominated before the sewage outfall was diverted, has thankfully kept a low profile. Such are the interests of those that study the phytoplankton.

The general principles that govern phytoplankton growth — light needed for photosynthesis, nutrients for the accumulation of biomass — are well-known. What Reynolds's book brings us is a quantitative appreciation of the things that limnologists have been generalizing about for decades. Take, for example, the problem of a population of diatoms slowly sinking through an unstratified wind-stirred lake. Reynolds shows by simple algebraic equations that in less than five times the minimum settling period under still-water conditions, 99% of the cells in continuously-stirred water will have reached the bottom mud. This surprising conclusion holds irrespective of the degree of turbulence. Here and elsewhere in the book equations are clearly derived for the biologist who can understand elementary mathematics even though he may not be good at doing it. Reynolds works with the same clarity and precision through the quantitative analysis of many other

physical and biological phenomena, including the changing light field, photosynthesis and productivity, the kinetics of growth and nutrient uptake, density gradients and terminal sinking velocities. Even apparently subjective topics, such as algal succession and population diversity, are given a similar numerical treatment.

One result of this rigorous quantifying is another alphabet soup, a text full of symbols; nearly a hundred are brought to order in the glossary. The basic units of the SI system are used in (almost) all the quantities, so that it is easy to build from one equation to the next and to follow even those with many terms. I found only a couple of dimensional errors.

The other strength of this book is that it recognizes the importance of knowing the special story of each alga's life cycle and predilections. For example, dinoflagellates are strong swimmers and do well on calm days in stratified lakes, apparently selecting just the right depth for their needs. Diatoms, which sink out like bricks under these conditions, enjoy the turbulence of early spring when every phytoplankton cell drifts at the mercy of the currents. Within the diatoms small differences in size and shape will determine such unrelated features as whether a cell can be re-suspended from the sediment, and how fast it will take up a particular nutrient.

The book contains a thorough treatment of much of the current knowledge on phytoplankton and what causes them to wax and wane in lakes. It is not just a derivative account but abounds with original ideas. The content is precise, and even pernickety (at every mention the word "alga" is in quotation marks, apparently because of its loose taxonomic meaning), but the style of writing is almost conversational and the book may be used for a good read as well as for reference. □

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● *Quaternary Extinctions*, reviewed in last week's *Nature* (p. 225), is distributed in Britain and the rest of Europe by Eurospan, 3 Henrietta St, London WC2E 8LU. Price £63.50.

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28-1-87
A. Galt

New skills for new technologies

from Richard Pearson

In some areas — biotechnology being an exception — employers requiring graduate skills seem reluctant to take on candidates with higher degrees.

THE United Kingdom, like many other countries, is living with a shortage of experienced information technology specialists while demand for these skills continues to grow. Whereas higher education has provided the basic skills and training for most of those entering the new technologies, the role of employers in training and developing new skills is now being emphasized. A UK government committee on the shortage of information technology skills has highlighted the need for a new "partnership between industry and higher education", and the University Grants Committee in its policy proposals for the 1990s stresses the key role to be played by "market signals" and the need for industry to improve its image if suitable students are to be attracted.

What information is available about the pattern of skills needed for the new technologies to help industry, education and students assess their training priorities? The United States has a vast array of studies and data about occupational trends, both past and future, as illustrated in last month's Employment column. In the United Kingdom, however, the only national forecast available does not provide anything like the necessary level of disaggregation: for example, it combines all scientists and engineers into a single category. Reliance therefore has to be placed on a series of *ad hoc* and often independently generated studies. What, then, do they reveal about the skills needed for the new technologies?

The term "new technologies" is, of course, a label of convenience and many of the constituent technical advances have largely been derived from existing technologies. Thus microelectronics is the miniaturization of electronic components, giving rise to major reductions in size, weight, power consumption and cost — the advantages which allow major new applications. Similarly, biotechnology has been used for centuries in brewing and fermentation, although it is the advances in genetic manipulation and bioprocess technology that have shown its potential in products as diverse as pharmaceuticals and feedstocks. Thus, although these technologies are "new", close examination shows that advances tend to be incremental; their contribution to society is determined as much by their application as by technological breakthroughs. The new technologies are found not only in the "sunrise" industries — they also have an important role to play in

revitalizing the "sunset" industries.

Employers' recruitment of staff to apply the new technologies similarly focuses largely on extensions of existing disciplines and emphasizes the key role of undergraduate degree programmes. The electronics industry relies almost exclusively on graduate intake to meet its future needs for professional staff, — the traditional entry routes, of promotion from craft or technician ranks, and part-time study having almost disappeared. Most employers are seeking graduates with broad-based electronics or related skills at a first degree level — even in highly specialized areas, such as semiconductor design and production, the preference is still for a majority of recruits from good quality first degree programmes rather than postgraduate courses.

The picture that emerges for information technology and computing is similar, although a large proportion of the intake to computing functions is of graduates of any discipline. In robotics, the demand is for graduates who can link mechanical, electronic and software skills, and not for fundamental science or a highly specialized degree.

Of all the new technologies, it is in biotechnology that postgraduate qualifications are most relevant. Again the disciplines sought are not new, but built around existing programmes. Here industry is much more closely linked to basic science, and few professional staff in biotechnology research have less than a PhD and, say, three further years of research experience.

Thus although the new technologies have a high dependency on higher education for their supply of skilled manpower, few of the skills initially sought are highly specific or dedicated to one type of narrow technology, discipline or employer. Rather, they allow for some flexibility in terms of course content and indeed a degree of substitution, for example, between applied physics and electronics. A further advantage is that these demands are also not numerically large when compared with an annual graduate output of around 100,000 per year — electronics and information technology each taking several thousand new graduates each year and biotechnology taking only a few hundred. These needs, however, are not being met.

The pattern and extent of shortages, as measured by vacancies that employers are unable to fill is, of course, greatly

influenced by past supply, present need and, particularly, the state of the economy. Thus the serious shortages of electronics and computing skills of 1978–80 abated during the recession but began to reappear in 1984. This year, the main areas of skill shortages have largely been restricted to electronics and computing staff with 3–7 years of experience, together with numerically smaller shortages in areas such as robotics, control, and telecommunications engineering and bioprocess engineering. There is, however, evidence that the incipient shortages of newly qualifying electronics and computing science graduates will soon increase. There are also many areas of work where existing specialists do not have the appropriate personal qualities or motivation to move into line management areas or the application of new technology, where there is a major need for technical skills.

Several initiatives to improve future supply are under way. The number of places on first degree courses in information technology is being increased for the second half of the 1980s while conversion courses are helping to alleviate short-term difficulties (see *Nature* 309, p100; 1984). Industry is also becoming increasingly involved with higher education through the provision of staff and equipment, and in course planning. A broader cross-section of students, however, must be encouraged to apply for the growing number of places, and especially those with the potential to apply such skills in line management and non-technical areas. Women represent a major untapped source of skills; they still fill less than 10 per cent of the places on information courses.

Improvements are already under way to increase the supply of key skills. However, an improved understanding of future manpower needs for all the new, and indeed existing, technologies is still needed to aid planners in government, education and industry, careers advisers and potential entrants. Such changing needs, and the different ways in which they might be met, such as retraining or substitution, also need to be regularly monitored and reviewed in a systematic way; and employment, education and training policies adjusted as appropriate if skill shortages are not to be a recurring problem in the future. □

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Confused comment on Warnock

The British House of Commons has had a muddled debate about the regulation of in vitro fertilization. The British government should devise more liberal legislation than was urged last week.

DAME Mary Warnock's report on *in vitro* fertilization and related matters seems to have had a better reception from the general press than from the British House of Commons, which spent last Friday (22 November) debating the issues that had been raised. By legend, this would have been one of those occasions when the British parliament should have been at its best. With nothing to be decided, and with no compulsion on party managers to dragoon their members through some predetermined voting booth (called a "division lobby" at Westminster), Members of Parliament with a philosophical turn of mind should have been free to take a broad view of an important set of issues, giving the government some durable help with the design of the legislation that it is committed to produce. In the event, the discussion was down-at-heel, dominated by complaints that the Warnock Committee failed to define the point in the development of an embryo at which life may be said to begin, by expressions of horror at what people in laboratories were about and by some simple misunderstandings that would be laughable if they were not held by those who will be responsible for whatever legislation eventually emerges.

It is inevitable that liberal opinions should be drowned in such a clamour. Even so, the view that the practice of *in vitro* fertilization is an important part of the treatment of infertility was strongly put. The case for research with living early embryos was put, but unavoidably made to seem simplistic by the apparent need to make its importance tangible by guessing at what the outcome might be. Surrogate motherhood won only the most tepid advocacy — most people are against it. The House of Commons seemed not to be especially downcast at the reminder (which Warnock vividly provided) that it has done nothing, these past twenty years, to deal with the legal problems occasioned by the now widespread practice of artificial insemination (where, under British law as it stands, such a child is illegitimate and should formally be adopted by its mother's husband, and where Warnock recommended automatic legitimacy but also disclosure of the method of conception at the child's majority). In the circumstances, the most memorable feature of the debate was that those in favour of the proposition that embryos are living persons from the instant of conception included both Mr Ian Paisley, the Ulster Unionist, and Mr John Hume, the leader of the Social Democratic Labour Party in Ulster — inveterate opponents on almost every other issue. The danger only barely concealed by the debate, in which the government promised that it may be possible to find administrative devices for regulating Warnock matters in the spell before legislation can be devised, is that some MP will jump in with a private member's bill drawn so emotively that nobody will dare oppose it.

What the House of Commons should have understood last week, and what the British government must now appreciate, is that there is a world of difference between the novel techniques in human embryology that are already parts of medical practice and the opportunities that have arisen in research. That the use of novel medical techniques should be regulated is not, of course, unusual and is not, in principle, objectionable. In Britain as almost everywhere, both abortion and (further from the clinic) adoption are regulated by law. Warnock asked that those offering *in vitro* fertilization should be registered, which is fair; there is also a case for asking that data should be securely logged, on which the committee was ambiguous. The committee also

agonized about surrogacy, concluding that the practice is always undesirable but that surrogacy with a commercial element is frankly unacceptable, and must be made illegal. Mr Norman Fowler, the Secretary of State for Social Services, warned those within earshot that surrogacy with payment may already be illegal under the British adoption laws as they stand. But even that argument is not as simple as it sounds, for there is an important difference between what may seem the sale of intact people and the prearrangement of a genetically known birth. Even on this contentious point, the British government should shy away from outright bans, as one of the minority reports of the Warnock Committee asked.

Research is quite a different kettle of fish. The Warnock Committee recommended licensed research with embryos no older than 14 days. The weakness of that position is that the time-limit is arbitrary. Moreover, the case for research on early embryos is necessarily sketchy — it is the old difficulty that people cannot tell what they will find until they look. The sensible course of action for the government to follow is to accept the Warnock proposal for a committee to oversee the conduct of all research projects proposed, much along the lines of the committees that have regulated recombinant DNA research in different places, but with the added proviso that a project should be shown not merely to be harmless but potentially valuable. The time-limit should be dropped. And the proceedings of such a committee should not merely be public but publicized. Then even the House of Commons might understand why the opportunities in this field are exciting and important. □

Bettering farm support

The Reagan administration should give agricultural research its due.

AS the Reagan administration began its curious effort last week to find budget cuts that will eliminate a \$200,000 million a year deficit without raising taxes, one of its first discoveries was the \$10,000 million or so spent each year to keep farmers in business. According to the "supply-side" dogma that still seems to grip everyone in the administration (except perhaps David Stockman, director of the Office of Management and Budget), supply creates its own demand; remove restraints on production (that is, government intervention), and everything will work just fine. Hence the not-so-surprising talk of doing away with the 50-year-old strategy of farm price supports in favour of what is being called a "market-oriented" approach.

The Reagan administration is not renowned for its grasp of history, so a quick refresher course on what happened the last time the United States tried *laissez faire* in farming is perhaps in order. Above all, it is worth remembering that throughout the 1920s, it was not just US farmers but farmers everywhere who struggled with chronic overproduction. During that decade of booming prosperity for business and industry, the farm economy was deathly ill. The prices of commodities, always subject to cycles of boom and bust, swung dramatically; the price of wheat, for example, fell from \$3 a bushel to \$0.30. By 1932, when the depression had finally struck the rest of the economy, the prices farmers received for their crops compared with the prices they had

to pay for the goods they consumed had fallen to half that of 1914. In 1929, more than a quarter of US farm income came from exports, again now talked of as the salvation of the US farmer. But then, far from being a salvation, export dependence precipitated further disaster when the export market collapsed.

Overproduction is still a chronic problem. And if, now, imbalance of general trade has at least provided Europe and Japan with the US currency with which to purchase US goods (not the case in the 1920s), the potential customers' restraints on trade to protect their own farmers and the overpriced US dollar are at least equal barriers. It is difficult to see how overproduction of US farm commodities can create its own demand in these artificial circumstances.

There is nevertheless much that could be done to reform the US price support system. The administration is looking in the right place, if in the wrong spirit. Whatever its ambitions now, the administration will probably not be able to eliminate the system of price support, which has at least served well to smooth out the natural cycles of boom and bust. The chief mechanism is that of loans granted to farmers at harvest time against crops as collateral; if the farmer is unable to sell his crop at a price equal to or better than the loan price, he has the option of defaulting, in effect selling his crop to the government. This prevents a price-depressing glut at harvest time and removes a minimal amount of overproduction from the market. But there is little justification for maintaining the expensive programmes that amount to all-out production incentives. Any rational farm policy must put a premium on greater efficiency rather than greater production. That is simply not the case now, when farmers find the best and, indeed, often the only avenue to increased income is expansion.

The present approach to agricultural research can only exacerbate these structural failings of the farm programmes. The emphasis on applied, production-oriented, short-term results brought about by the anachronistic formula-fund system of agricultural research can only encourage the overproduction mentality. By every estimate, US agricultural research is woefully undersupported, yielding a return on investment many times greater than the norm for other businesses. The absence of peer review in the award of most research grants ensures that special interests have a disproportionate say in the choice of research priorities, exacerbating the short-term view of many researchers in the land-grant college system.

Yet a greater investment in fundamental research, particularly plant molecular biology, could lead to substantial reductions of production costs, for example by reducing or eliminating the need for energy-intensive inputs such as pesticides and fertilizers. The \$46 million that Congress managed to appropriate this year for research is a sound first step, but far less than private corporations are spending in this field. Now, within the overall context of US farm policy, the time has come to acknowledge that agricultural research is too important to be left to the vagaries of share-the-wealth formula funding, and more relevant to the long-term stability of the farm economy than the narrow pursuit of increased production that now passes for a research strategy. □

European initiatives

The European Science Foundation has had ten modest years. Now it needs to be more daring.

THAT it is high time that something more was done about the organization of science in Europe is beyond dispute. What perplexes people is to know with some clarity what should be done. Lord Flowers, the first president of the European Science Foundation, canvassed one modest set of proposals at the tenth anniversary meeting of the foundation in Strasbourg last week. In a public lecture on "the first twenty years" (a joke intended to encompass both the formal lifespan of the foundation and its gestation period), Flowers rightly claimed on behalf of the foundation a string of notable achievements in the past few decades, among which (just now) the most conspicuous is the plan for building a powerful source of synchrotron radiation in

Western Europe (see *Nature* 1 November, p. 3 and 15 November, p. 189). Properly, however, Flowers boasted of the way in which the foundation has been asked by the Council of Europe to carry out a study of the ways in which the mobility of scientists in Europe might be increased and he urged that the British government's last-minute proposal at last month's European meeting in Paris, that there should be a European academy of some kind, should be taken more seriously than its half-baked character would otherwise ensure. Persuasively, he argued that an institution such as the European Science Foundation, which is essentially a forum in which European grant-making agencies can put their heads together over common problems, and think of solving some of them, must run the danger of losing touch with the individual researchers on whom its success must depend.

Nobody in Europe will quarrel with a word of this, at least so far as it goes. Lord Flowers, the chief architect of the foundation, is understandably wedded to the notion of organic growth in European collaboration. He and others were also at the outset convinced that the creation of a body such as the European Science Foundation beneath the umbrella of the European Communities would be disastrous, however large the annual budget. That decision was without question correct. Freedom from the Commission's tutelage has allowed the foundation to draw its membership from all over Europe, not simply from that part in which the European Commission's legal writ runs. More important, the foundation has not found itself in the invidious position of having to look for solutions to the short-term and often artificial problems that the Commission repeatedly singles out as urgent. If it had accepted the Commission's offer of financial support, the foundation would long since have found itself tackling the impossible task of hammering out, or even carrying out, a research programme to make European computers better than other people's, or to resolve the endless (and intrinsically political) conflict about the basis on which environmental regulations should be set. Instead, the foundation has been able to tackle the tasks at which its chance of success was reasonably good. The long-term goal is the encouragement of a sense of community within European science. With the passage of time, the argument seems to run, the scientific community will itself determine how a European scientific community should be organized.

If there were the whole of time to play with, such a policy would make good sense. But the successful pursuit of the ideal is possible only within an ideal environment. In Europe as it really is, the problems afflicting the scientific communities of at least half a dozen states are too urgent to be allowed to wait. When European research institutes such as the European Organization for Nuclear Research (CERN), rightly held out as successes for European collaboration, are themselves threatened by the possible defection of member states such as Britain, when plans for collaborating in other international research programmes (such as deep-sea drilling) are frustrated by the general shortage of funds and when it seems likely that important pieces of nationally-built scientific equipment will be wasted because their builders (the British research councils in particular) can no longer afford to use them properly, there is a strong European case for some more robust mechanism for mutual support in times of trouble. For its own sake, the European Science Foundation does not want to be hurried. But that may be too great a luxury to expect.

What, in these circumstances, might be done? The best chance is that the foundation may be able to move on from identifying barriers to mobility within Europe to becoming a monitor of what is happening within European science. There are housekeeping jobs to do, such as the compilation of the up-to-date register of who does what research, now done by some European governments, but always unsystematically. There is also the more contentious task to tackle of inducing among the European research councils (most of whom are members of the foundation) a sense that coordination of some kind would be desirable. Even a device as simple as trying to produce an annual report on European academic science would be a sensible starting point. But these are only beginnings. □

Italian research

New president's ambitions firm and unclouded

Rome

PROFESSOR Luigi Rossi Bernardi, the respected haemoglobin physiologist who is the new president of the Italian national research council (CNR), is moving like a whirlwind through the dusty corners of this dominant institution of publicly-funded science in Italy. But some old hands are already wondering whether the wind will really clean up the moribund organization or merely raise dust.

Whatever the outcome, the new president is starting as he means to go on, not least by introducing real (if presently crude) measures of output and scientific quality into the CNR system, where many groups and projects, critics say, are continued long beyond their useful life. Thus, within days of his appointment, Rossi Bernardi obtained tapes of world publications and citations from the Institute for Scientific Information in Philadelphia, and began analysing them on a computer in Milan for the publications of CNR groups and institutes.

The analysis took a week of computer time, but threw up results such as that some ten CNR groups or institutes (out of 270) had published nothing for five years. Such groups will be closed, Rossi Bernardi promises.

And at an extraordinary meeting last week in Rome, nominally to present the annual report to the assembly of CNR which was in practice the new president's first chance to present his policies publicly, these policies were alternatively vigorously supported, barely criticized and finally approved. According to one enthusiast, who spends half his time at the US National Institutes of Health, the occasion was fundamental in that it represented a thorough changing of the old guard.

As if to emphasize the political nature and certainly the importance of the task ahead, Rossi Bernardi was flanked throughout last week's meeting by the minister of research, Mr Luigi Granelli, who seems at least as determined as his new CNR president to change the face of Italian science. It was the first time in the memory of some observers that a minister had spent so long at a CNR meeting — which ran into a second day, with a seminar on the international relations of the council. In that half of the meeting, distinguished Italian scientists working abroad presented their comparisons of foreign (often American) procedures with those in Italy, thus — inevitably — lending weight to Rossi Bernardi's own proposals. Italian science attachés from Washington, Moscow and elsewhere were also afforded their own time for criticisms. Rossi

Bernardi later made some "quite exciting proposals", according to one attaché, for the revitalization of the attaché network — thus emphasizing the importance the new CNR president attaches to avoidance of parochialism.

Granelli also pledged himself to removing the "parastatale" status of the CNR, which equates CNR scientists with the meanest of Italian civil servants, and whose effect is to make promotion entirely dependent on age. He also estimated present Italian research and development spending as 1.3 per cent of gross national product (it was under 1 per cent at the end of the 1970s), and set a target of 2.5 per cent by the end of the 1980s. There was a 40 per cent increase in public spending on science in Italy last year, it was claimed.

Rossi Bernardi's objectives for 1985 include:

- Decentralization.

- Cutting a tangle of red tape and obscurity in CNR financial management, including a new separation of administrative and research expenditure.

- Setting up a databank on CNR personnel.

- Monitoring scientific output.

- Better control of the "finalized projects" (through which, in practice, many university groups raise funds for only nominally applied research projects).

According to the minister of research, such reform is necessary not only for CNR, but should act in CNR as a "nucleus" for the reform of science in all Italy.

All stirring stuff — but some sceptics in Rome last week felt the momentum would finally dissipate in the treacle of Italian bureaucracy, while others felt Rossi Bernardi to be well-meaning but politically naive in a country where politics is all. Granelli, who is strongly behind the new CNR president's initiatives, may also not last long — few Italian ministers do, as governments fall frequently, or he may be promoted — when Rossi Bernardi may find himself exposed and rather alone. But as Granelli himself said last week, Italy faces "marginalization" if research is not taken in hand.

Robert Walgate

NIH research

Reducing grant-writing burden

Washington

THE US National Institutes of Health (NIH) are to consider extending the durations of their extramural research project grants in order to reduce the burden of preparing grant applications and to provide reviewers with better evidence on researchers' performance. This general proposal was well received at a meeting last week of the NIH director's advisory group, and NIH director James Wyngaarden said after the meeting that he took the favourable response as an "endorsement in principle" for extending grants to first-time applicants beyond the usual 3 years. Grants to mid-career and distinguished investigators might also be extended.

The move comes in response to growing concern in the academic community that the pressures created by the system as at present operated — termed "intimidating" by Wyngaarden — are impeding research creativity. Most first-time grant holders must start preparing renewal applications after less than 18 months' work — hardly enough time for a realistic appraisal of progress. Furthermore, researchers often spend up to three months drafting applications, because most believe, probably correctly, that their chances of a renewal depend on the result of a detailed scrutiny of the proposal.

Several invited participants from academic institutions last week urged a return to the philosophy of investment in promising scientists, rather than "procurement research". There was dissatisfaction that

"study groups", the first level of peer review for grant applications, spend too much effort examining minutiae and "micro-managing" projects. Many suggested that reviewers would be able to make more informed judgements if most first-time grants were to be extended to, say, five years. One proposal that Wyngaarden found attractive was that grants might specify a fixed amount of money that could then be spent over a longer or shorter period depending on progress. This solution might allay the anxiety of having to produce results within three years, while avoiding substantial extra commitments on the NIH budget. However, no formal vote was taken, and Wyngaarden stresses that any change to present practice will first be subject to internal review.

Dr Joshua Lederberg, president of Rockefeller University and a leading critic of present practice, said it was not yet clear how any change should be implemented, and stressed the need for more information on the careers of those who fail to obtain grant renewals and disappear from NIH records. Academic institutions are becoming less able to find alternative work for such people, and the average duration of tenure of a trained researcher within the extramural system is only seven years, which Lederberg thinks represents a wasted investment. Reviews emphasizing the track record of the applicant would, he feels, bring much-needed stability to the careers of mid-term investigators.

Tim Beardsley

European air pollution

Commission's directive in balance

Brussels

UNLESS they can hammer out several major political compromises, environment ministers of the European Communities meeting here next week (6 December) have little chance of getting to grips with the problem of air pollution.

For the past several weeks, discussions of the draft directive on limiting emissions from large combustion plants have shown little sign that the member governments are prepared to relax the positions they defended at the last council in June. If anything, they seem more firmly entrenched, at least to judge from the number of objections lodged to this important and controversial piece of Community legislation.

The Commission wants to see air pollution programmes in force by 1 January 1985. The draft directive would set uniform emission standards for sulphur dioxide, nitrogen oxides and dust from plants with a thermal output of 50 megawatts or more. Its aim is to reduce, by 1995, the volume of sulphur dioxide emitted by 60 per cent (compared with 1980), and dust and nitrogen oxides by 40 per cent.

The member governments, however, remain divided both on the particular emission limits in the draft directive and on the principle whether there should be global emission standards at all.

In the forefront of opposition to the directive is the United Kingdom, which argues that deadlines are not necessary because scientific evidence on the contribution of industrial plants to air pollution is still incomplete, and that the cost to industry of implementing the directive would not be offset by its effects on the environment. According to the Commission, the extra cost of fitting new combustion plants with desulphurization equipment to remove 90 per cent of sulphur from fuel gases to achieve the limit of 400 mg of sulphur dioxide per cubic metre would be US\$100 per kilowatt of electricity produced.

Other opponents of the directive are Greece and Ireland, both of them conscious that their industrial emissions are likely to increase in future. Greece is planning new generating plants using its own indigenous and particularly polluting fuel. Ireland, for this half-year the president of the council, is in a ticklish position, having to show itself in a forceful role while having to replace much natural gas usage by coal-fired electricity generating plants.

Luxembourg, which has no plants that would now be affected by the directive, is also worried about the future, while Italy, which also opposes the directive, is asking that emission limits should be fixed nationally, not at Community level.

The emission limits proposed by the Commission are, however, considered too

weak by West Germany and the Netherlands. Denmark, which is in favour of the directive, also considers the emission limits too strict, especially for nitrogen oxides and dust.

Indeed, several governments would prefer to see dust left out of the directive altogether, partly because monitoring of dust emissions can be up to 50 per cent in error and because of the lack of data about present practice. The British want the directive to apply only to steam or electricity generators and not to the cement works, brick works and/or treatment plants that would at present be included. While some (including France, Ireland, the Netherlands and Italy) consider these a dangerous source of nitrogen oxides, other governments insist that control technology is at present inadequate for the directive to be effective.

There is also disagreement about the size of plants to which controls should apply. The United Kingdom has proposed a 100 MW threshold, Ireland 150 MW. Although the Commission's proposal includes a number of temporary exemptions, as when costs of compliance would be disproportionately high or when total annual emissions are lower than in other countries, even these are considered insufficient by countries such as Greece, Ireland and Luxembourg.

The Irish presidency's search for a compromise is turning out to be difficult. Three proposals have been put forward.

- Limiting the directive to new plant, which West Germany fears would allow too much plant now planned or being built to escape regulation by the time the Community directive is turned into national law.

- Raising the threshold to 150 MW, to which some object that the result would be more small plants and thus more pollution in total.

- A quota system with national emission rates fixed on criteria such as the level of energy consumption and the contribution to transboundary pollution. Belgium objects on the grounds that this compromise would be discriminatory and West Germany on the grounds that it violates the polluter-pays principle. The Commission itself fears that this compromise would conflict with Community law.

At next week's meeting, the ten governments will be equally divided, with Britain, Greece, Ireland, Italy and Luxembourg in opposition. A further complication is that the Commission's draft directive has been severely criticized by the European Parliament on the grounds that it is too lax. Ursula Schleicher, West German Member of the European Parliament, for example, says that the five-year exemption for plants below 100 MW will generate the building of small plants. She also advocates a

supra-regional monitoring system for air pollution and financial support from the Community to deal with regional problems.

The European Parliament itself has called for the application of the standards to existing plants and for stricter emission values by the end of 1990.

Another contentious issue next week is that of phasing lead out of petrol (see *Nature* 4 October, p.401). The council will be able to take a decision only in principle, because the European Parliament's opinion has been blocked by the 158 amendments submitted during its last session.

Denmark, Luxembourg and the Netherlands have, however, joined West Germany in their willingness to introduce lead-free petrol by 1986 while others would agree that this date should be optional (with the exception of Greece, which is unhappy about the whole thing). France is saying that the interim standard of 0.15 grams of lead per litre of petrol need not be included in the directive.

Among the other issues that will be discussed next week are a proposal that Community governments should aim at US emission levels from automobiles by 1986 rather than 1988, but again there are differing opinions about the need for an interim set of emission standards. The meeting will also have to decide whether to spend 100 million ECUs on a five-year programme to protect forests from fires as well as acid rain, chiefly by setting up a monitoring network.

Anna Lubinska

New climate unit

A NEW centre for atmospheric and oceanographic research was opened at the University of Oxford two weeks ago by the chairman of the UK University Grants Committee, Sir Peter Swinnerton-Dyer, in his capacity as chairman of the Meteorological Committee, which answers to the Secretary of State for Defence. The new centre, which promises to play a significant role in both satellite meteorology and large-scale oceanographic-climate modelling, is unique in being administered and supported by the Meteorological Office while being located in a university department — Oxford's department of atmospheric physics.

Most of the staff at the new unit are drawn from the Meteorological Office, with the addition of Dr Adrian Gill, a theoretical oceanographer (who until recently was at the University of Cambridge). The staff are drawn equally from the fields of numerical oceanography and satellite meteorology, and the expectation is that close proximity to the atmospheric and remote sensing expertise at Oxford will focus and stimulate research on such problems as tropical climate-ocean interactions and satellite sea-surface measurements.

Philip Campbell

Gene therapy

US clinical trials imminent

Washington

THE way is now being cleared for the first human gene therapy experiments, which could take place as early as next spring.

One research group, headed by Dr Ted Friedmann of the University of California at San Diego, has already received permission from the local Institutional Review Board (IRB) — the panel charged with protecting human research subjects — for clinical trials of a procedure aimed at correcting the genetic defect responsible for Lesch-Nyhan disease, although the proposal would still have to be approved by the National Institutes of Health Recombinant DNA Advisory Committee (RAC) before trials could begin.

In anticipation of Friedmann's and other proposals, RAC earlier this year established a working group on human gene therapy which is in the process of drawing up guidelines that will tell researchers what information to submit with their proposals. The working group, which includes ethicists as well as physicians and researchers, will also screen all such proposals before presenting them to the full RAC for consideration.

A draft of the guidelines, known as *Points to consider in the design and submission of human gene therapy protocols*, makes it clear that RAC plans to look beyond the questions of risk and protection of human subjects in its scrutiny of proposals. Many members of the working group strongly favour expanding RAC's purview to include such social issues as whether the procedures will be subject to proprietary rights and whether the proposed somatic-cell treatment could affect the reproductive cells of the patient.

Nevertheless, it is considered unlikely that the working group or the full RAC will turn down legitimate proposals from researchers seeking to perform somatic-cell gene therapy. One scientific member of the working group has in fact resigned in impatience at needless hand-wringing over a foregone conclusion.

Meanwhile, a broad consensus appears to have emerged among religious leaders and ethicists that although germ-cell therapy — that is, alteration of heritable traits — raises difficult ethical problems, no such objections exist in the case of somatic-cell therapy. The Office of Technology Assessment (OTA), which is releasing a study on human gene therapy next month, found strong support for this view among a group of theologians, religious leaders and ethicists it assembled for a meeting in September.

The Food and Drug Administration (FDA) is also preparing itself for the first gene therapy experiments. In an announcement to be published shortly in the *Federal Register* (and which will be part of a larger statement of overall federal policy on the

regulation of biotechnology being coordinated by the Office of Science and Technology Policy), FDA will make clear that it intends to view recombinant DNA used for human gene therapy to be "biologicals" subject to FDA regulation.

Before beginning clinical trials, researchers would have to apply to FDA for an "investigational new drug" permit for the recombinant DNA; and FDA would have to approve the product's safety and effectiveness before its general use could begin. (Unlike the RAC guidelines, which are technically binding only upon research institutions that receive federal funds for recombinant DNA research, the FDA procedures have the force of law.)

The OTA report notes four research groups that are close to clinical trials. According to NIH officials, at least one

Japanese telecommunications

Future goes up in flames

Tokyo

A LITTLE forgetfulness on the part of a Tokyo telephone repair man last week almost certainly caused Japan's first "telecommunications disaster" — and a heated debate about the vulnerability of the new "electronic society" into which the nation is rushing headlong.

What the repair man apparently forgot to do was to remove a small piece of rag wrapped around telephone cables in a lead pipe he was soldering. The result was a fire that spread into underground ducting and destroyed 90,000 telephone, databank and telex links, cutting off telecommunications in one whole ward of Tokyo and bringing a large part of the nation's banking operations to a halt.

Worst hit was Mitsubishi Bank, the nation's fourth largest. The fire cut the vital link between its central mainframe computer, which holds details of 6 million customers' accounts and records 200,000 transactions a day, and the office that relays the data to its 217 branches. All Mitsubishi's banking operations throughout Japan immediately came to a complete halt. Three more of the nation's top five banks also lost links with central computers and had to stop transactions in and around Tokyo, and 48 post office branches close to the fire had to halt savings account operations.

Businesses in the area were thrown into confusion. One of the big security companies had to stop trading when its computer was cut off, a major parcel company lost access to the central computer that records the routing and delivery of the 600,000 packages it handles each day, hospitals lost access to medical records normally sent by facsimile, the major overseas news wire service lost contact with

grant application recently received by the genetics study section includes a proposal for human trials. All three of the target diseases mentioned in the OTA report as likely early candidates involve single gene defects that result in enzyme deficiencies.

One candidate is Lesch-Nyhan disease, recognized by renal failure and central nervous system disorders. The others are purine nucleoside phosphorylase deficiency and adenosine deaminase deficiency, both recognizable by the severe immunodeficiency they cause. Stuart Orkin (Harvard University) and David Martin (University of California, San Francisco, in collaboration with Genentech) have set their sights on genetic replacement of the deficient enzymes.

In each case, the defect is in the bone marrow; the most likely clinical approach would be to remove the patient's marrow, infect the cells with "repair" DNA carried by a retro-virus, and then replace the marrow.

Stephen Budiansky

foreign offices, and hundreds of retail and wholesale businesses saw telex, credit card and telephone orders vanish and supermarkets unable to order fresh stock.

Residents of Settagaya ward, where the fire broke out, slipped back into the pre-telephone era. All telephone lines were cut, including those for emergency services, so that people had to report crimes and fires to local stations on foot. To try to see things through, the telecommunications monopoly, NTT, brought in armies of couriers to relay messages between the central telephone office and local residents — four days after the fire, nearly 5,000 messages a day were being carried. A satellite link was provided from a mobile transmitter, a mobile exchange brought in and 800 public telephones set up in the street — some 30 minutes of queuing was, however, needed to make a call.

Frantic efforts by NTT to restore telecommunication services have continued, with 10,000 man-days of work in the week since the fire. Telephone lines should soon be fully restored, although it will be another couple of weeks before all the damage is repaired.

Meanwhile, businesses in the affected area are finding to their chagrin that provisions for compensation are hopelessly out of date. An appeal to NTT by 600 shop owners on the grounds that "telephone paralysis is a new kind of city disaster" is not likely to meet with a generous response. The public telecommunications law, adopted around 30 years ago, provides only that if a telephone service is stopped for more than five days, compensation will be provided at the same rate as the basic rental charge: that means that compensation would reach about £20 after a three-week stoppage.

Alun Anderson

Informatics

Olivetti bids to rival IBM

Milan

OLIVETTI, the Italian-based typewriter and office furniture company now well-launched into a transformation into an informatics business, aims to become Europe's IBM, said economic studies director Bruno Lamborghini at a meeting at Olivetti's headquarters at Ivrea last week. But the company has a long way to go. It ranks tenth among world informatics technology companies, and is second in Europe only if it includes sales of electronic typewriters. Moreover, first place in Europe is taken by IBM itself, with seven times Olivetti's European sales. But Olivetti is chasing fast: its consolidated profits in 1983 were triple those of 1982, and its sales volume curve is at present rising like the graphs normally seen only in cartoons.

The rub for Europeans, however, is that Olivetti's rise and rise is only partly a European phenomenon. Although nobody would say so explicitly, it is clear that Olivetti has written off any major prospect of growth by combination with other European partners. Instead, it is relying on the deal made earlier this year with a big wolf, the American Telephone & Telegraph Company (AT&T, formerly Bell Telephone), which has injected capital into Olivetti to a total now reaching a quarter of the outstanding equity.

The deal allows the fraction ultimately to reach 40 per cent, coming close to control, but in a display of bravura occasioned by Olivetti's tremendous success lately under its dynamic president, Carlo de Benedetti, the company shows no fear that it will act merely as the back door by which the American wolf will enter Europe.

Indeed there is talk in Ivrea of showing AT&T how to make proper use of its wasting asset, Bell Laboratories, which are seen as "good for Nobel prizes" but relatively weak on applications technology for the new informatics market. Olivetti now has a hundred or so people at Bell Labs at any one time, where they have access to all but defence and public switching work. This offers Olivetti "a unique opportunity", says Lamborghini. The company has always been conscious of design, which in turn means marketing, which in turn means the needs of office users — which is where the information revolution is taking place. The combination of Bell research (and communications technologies) and Olivetti's closeness to the office could be a very powerful combination that would leave mainframe dinosaurs such like IBM behind. Such is the stuff of dreams at Ivrea.

But what of combination in Europe? "The political and economic discord that currently divides the European countries does not permit optimism on possible progress in the immediate future", write

Lamborghini and the corporate marketing director Vittorio Cassoni cautiously in a joint report. Esprit, the joint European project on fifth generation ("intelligent") computers, is welcomed at Olivetti but seen as "very small" compared with the efforts of US manufacturers, said Lamborghini last week.

It is playing an important role in the definition of common European standards, however. Esprit provides a point of contact where companies can meet ("it was incredible how the European companies, even in the same country, were isolated") but there is frustration in Olivetti at how national and inter-company rivalries are hindering the project. Thus Olivetti directors, asked whether the company has decided to go it alone (with AT&T) without the help of Esprit, shrug their shoulders in a gesture of "what do you expect?", and smile.

European Commission

Golden parachutes at Ispra

SOME 120 high-grade scientists and technicians earning between £25,000 and £40,000 a year at the European Commission's Joint Research Centre (JRC) at Ispra, in Italy, are to be offered early retirement on terms starting at 70 per cent of their current salary. Or so says a proposal now before the European Parliament for its "opinion". According to Glyn Ford, Labour Member of the European Parliament and once science policy lecturer at the University of Manchester, that opinion should be "no" — although the Energy, Research and Technology Committee to which he belongs has voted the measure through.

Ford says the objective of the proposal is "perfectly sensible", for it is aimed at shifting 50–60-year-old nuclear physicists who were given lifetime appointments when JRC began as a Euratom research centre 25 years ago, and bringing in younger, more junior scientists in new disciplines. The European Parliament and the Commission have both been urging JRC to rejuvenate the shift to non-nuclear technologies and environmental issues, and the retirement scheme is a response to that.

"But I object to the quantity of cash involved and the method by which people are being retired", said Ford on Monday. The retirement scheme will be voluntary and unrestricted, "so only the best people will go". There has long been doubt about the scientific productivity of JRC, and this could make the situation worse, Ford believes. The result of a similar scheme at Manchester, Ford says, meant that all the people who could get jobs, and were in demand (such as computer scientists and accountants) left, depleting the university

As for research directions, Olivetti is interested in Bell Labs' flat screen (plasma) technology, in non-impact (thermal and ink-jet) printing, and above all in voice input and output. Artificial intelligence (AI) is also seen as "crucial and strategic", requiring research at many levels and in many directions, as intermediate products — particularly involving voice recognition — will be affected by intermediate developments in AI research. But Olivetti does not intend doing basic research in AI — it will leave that to Bell Labs.

As for products, Olivetti research director Arnaldo Pasini foresees a laser printer with all the graphic capabilities of a newspaper, a digital reader (to record incoming letters etc. in bit image form, so there will be no need to keep paper files), and vocal input and output, all within a few years. Most innovative technology will come from the United States, where Olivetti has now invested in some 20 venture companies, Pasini says. Olivetti's job will be to match it to the market: to become, says one commentator, "the great transformer".

Robert Walgate

of a useful cadre of people. Moreover, although those taking retirement from JRC will be obliged to notify the centre if they subsequently begin earning enough to take their total income (including pension) beyond their original income, the scientists come from many different countries and violations will be difficult to trace. The result will probably be a brain-drain "to our competitors" financed by the European Community, Ford claims.

JRC director Jean-Albert Dinkespieler, however, puts the matter in a slightly different perspective. The net cost to JRC will be 1.5–2 million ECU (about £825,000–£1,100,000) a year for ten years, only 1–2 per cent of the JRC salary bill, when account is taken that money will be saved by replacing highly paid by younger less well-paid people. Moreover, he points out, all new appointments will be on a contract (5-year renewable) basis, to avoid a repetition of the problem now being faced. Similar schemes have operated successfully at the European Space Agency, he claims. The researchers were too old to change speciality, and too young to retire, so what else should be done to create movement?

It would probably be a one-off exercise, said Dinkespieler, and would involve recruitment of biologists (an innovation for Ispra), air and water chemists and oceanographers for pollution studies. The Council of Research Ministers has given its outline approval to the scheme, he said, but European parliamentary approval (which involves the advice of many committees including Energy, Research and Technology) was also necessary. Ford's intervention had made matters more difficult.

Robert Walgate

Australian nuclear policy

Non-nuclear election party

Canberra

THE Australian Labor government's uranium export policies and the combined presence of joint US-Australian defence facilities at North-West Cape, Nurrungar and Pine Gap are looming large as issues in the forth-coming federal elections to be held on 1 December. Perhaps the most unexpected development in the run-up to the election has been the swift rise to prominence of a new single-issue political party — the Nuclear Disarmament Party (NDP), formed only in June, which appears likely to capture at least one seat in the half-Senate election, if recent polls are to be believed. The polls also suggest that the government will retain power in the lower house, with the personal popularity of the Prime Minister, Mr Bob Hawke, a large factor.

The Senate (the upper house), however, is the Achilles heel of the Labor Party, and triggered the downfall of the Whitlam government in 1975. In a Senate in which the government may again not gain control, NDP says that if elected, its members will vote only on questions relating to uranium, disarmament and the joint facilities. Ironically, this would limit their capacity for log-rolling deals, since the conservative opposition parties are likely to vote with the government on most nuclear issues. Mr Hawke said that a vote for NDP would be wasted and hastened to point out the Labor government's achievements in the disarmaments sphere. He added that, as the South-East Asia/Pacific region member country "most advanced in the technology of atomic energy including the production of source material", Australia has a designated seat on the board of the International Atomic Energy Agency and has recently become a member of the United Nations Security Council.

Since July 1983, Australia has been represented in international forums by an ambassador for disarmament, Mr Richard Butler, and funds have been made available for a peace studies centre at the Australian National University, in support of the comprehensive test-ban. As well as attempting to help solve the procedural problems that have plagued the conference on disarmament, Australia is also playing a large role in the current technical trials of an international test-ban verification network, run by the Geneva Group of Scientific Experts on Seismic Events (GSE). Coordinated from the Bureau of Mineral Resources in Canberra, Australian seismic stations at Alice Springs (Northern Territory), Narrogin (Western Australia), Charters Towers (Queensland) and Mawson (Antarctica) transmit data to Melbourne for relay to the other centres (Washington, Moscow and Stockholm) through the World Weather Watch telex system.

But the disillusioned ex-Labor supporters and young people of NDP reject the government's whole nuclear package. They believe Australia's best interests lie in closing the joint defence facilities and in leaving Australia's large reserves of uranium in the ground. **Jeffrey Sellar**

European all-change

PROFESSOR Eugen Seibold, president of the Deutsche Forschungsgemeinschaft, was appointed president of the European Science Foundation at the tenth annual meeting of the foundation in Strasbourg last week. Professor Seibold succeeds M. Hubert Curien, president of the foundation since 1980, who has felt it necessary to resign on his appointment as Minister of Research in the government of France. Professor Seibold has been active in the foundation's activities since its inception, recently as vice-president.

The Strasbourg meeting last week also appointed as the foundation's secretary-general Mr Michael Posner, until two years ago chairman of the British Social Science Research Council. Mr Posner succeeds Dr John Goormaghtigh, secretary-general at Strasbourg for the past six years.

The members of the foundation last week agreed that the budget for the coming year should amount to just under FF10 million, an increase of some 9 per cent compared with the previous year. In addition, the members of the foundation, mostly grant-making research councils, have agreed to contribute an equal amount to the cost of the foundation's "additional activities" — cooperative research projects to which interested members contribute and in which they usually participate. □

Acid outback rain

Canberra

ACID rain in the outback of Australia? Surely not. Yet this is the conclusion of two independent teams of atmospheric scientists on the hydrogen-ion concentrations in rainwater at sites hundreds of kilometres apart in Australia's Northern Territory. The teams, led by Dr Gene Likens of the Institute of Ecosystem Studies of the New York Botanical Gardens and Dr Barry Noller of the Office of the Supervising Scientist, Alligator Rivers Region Research Institute, presented their results at an international conference on the scientific application of baseline observations of atmospheric composition, held earlier this month at the Commonwealth Scientific and Industrial Research Organization (CSIRO) Division of Atmospheric Physics in Aspendale, Victoria.

Both groups found that at their respective rainfall sites at Katherine, 200 km

Yugoslavia

Brain drain

AN "irresponsible attitude" towards science and scientists has produced a massive brain drain from Yugoslavia, according to a new survey from the Zagreb Centre for Migration Research, reported by the Belgrade daily *Politika-Ekspress*.

According to the latest figures, during the past two decades some 5,000 Yugoslav specialists have left, with a peak of 1,371 leaving in 1968-73. The basic reasons, according to *Politika-Ekspress*, are "bureaucratic harassment", inadequate opportunities for promotion, or even the total lack of a job, and the desire for higher earnings and a higher standard of living. Vujica Jevdrevic, an expert in hydrology employed by the United Nations, who could not obtain "adequate conditions to continue his work" in Yugoslavia, and decided to remain in the United States. The "celebrated mathematician". The nuclear physicist Bogdan Maglic was fired from his Yugoslav post as a result of a bureaucratic error, while he was in the United States doing advanced studies, so that he decided not to return.

According to *Politika-Ekspress*, the brain drain is motivated by practical and economic considerations, and there are (implicitly) no political factors involved. Indeed, it is noted that "the highest political bodies and the Yugoslav leadership" have recently been demanding that the "irresponsible" stance of the bureaucrats towards science and scientists should be substantially reformed. It is noteworthy, however, that the peak years of emigration, 1968-73, cover the purge of scholars associated with the Matica-Hrvacka movement in Croatia, and a subsequent similar purge of intellectuals in Serbia. **Vera Rich**

south-east of Darwin, and Jabiru, 300 km east of Darwin, the average hydrogen-ion concentration was about one-third that of the corrosive acid rains of northern Europe and North America. The difference is that the sulphate and nitrate ion concentrations are at least an order of magnitude less than the Northern Hemisphere measurements and that the high hydrogen-ion concentration is accounted for by the unexplained presence of formic, acetic and other weak acids. At some sites, pH readings as low as 3 have been recorded. The organic acid concentration is highest in the early part of the wet season (November-December) and lowest in the monsoon period (January-March). Dr Likens is reported to have been keen to undertake the 30-month study in order to determine what atmospheric chemistry was like "before the Industrial Revolution".

Jeffrey Sellar

Creationism in confusion?

SIR — It was, perhaps, too much to expect the founders of the "Association for the Protection of Evolution" (APE) to give an objective report on the recent Biblical Creation Society (BCS) conference at High Leigh (News and Views, 25 October, p. 703). What is surprising, however, is that you should give such extensive and uncritical exposure to views which were self-evidently biased.

The very heading to your report, "Creationism in confusion", betrays a tendentious purpose, namely to present BCS in the worst possible light. Every difference of opinion or continuing debate among creationists is represented as evidence of "confusion" rather than the product of honest enquiry and developing ideas. By the same token, would the APE-men agree that evolutionary theory is "in confusion" because neo-darwinians, punctuated equilibriaists and reformed cladists disagree? Debate and disagreement is the stuff of scientific and intellectual progress and the BCS open day demonstrated that (contrary to the impression given) creationists are capable of constructive self-criticism at the highest scientific and theological level.

The conference report contains several serious errors of understanding. Howgate and Lewis fail to grasp the purpose of David Tyler's lecture which was not to reinterpret geology in terms of catastrophism but to illustrate from field observations (1) that the straitjacket of Lyellian uniformitarianism is a hindrance to the science of geology and (2) that there is a need to test the appropriateness of modern analogues, whether catastrophic or non-catastrophic.

Dr Darnborough, described as "BCS's top geneticist" is, in fact, a molecular biologist. He does not "believe in evolution". His paper argued, rather, that the operation of accepted evolutionary processes at the molecular level upon genetically perfect *created* organisms would lead to a living world with genetic characteristics essentially similar to those observed in modern organisms. This paradigm is fully consistent with both the scientifically observed facts and the biblical doctrines of creation and the fall. It further avoids the need to invoke the vast improbabilities of chemical evolution.

My own lecture "Christ and the Cosmos" is represented as a "keynote theological policy statement" and a condemnation of "American creationists". It was nothing of the kind. It was a positive exposition of an important New Testament passage which sets out the relationship between Christ and the created, divorcing "creationism" from the Creator, and illustrated this by reference to certain trends in American creationism. To suggest that I "condemned" or "attacked" the whole US creationist movement is so far from the truth that I am tempted to attribute to your reporters the unworthy motive of using

your journal to sow international discord among creationists.

The report continues with the statement that "a stalwart of the rival creationist organization the Creation Science Movement attacked the backsliding into theism of the BCS". First, there is no rivalry between BCS and CSM. The two societies have somewhat different aims but remain in close and amicable consultation. Second, your correspondents should learn a few basic theological terms before venturing into print on such subjects. Both BCS and CSM are proud to be "theists". It is "deism" that we, equally, disown.

Finally, we are astonished that you should include, as part of the conference report, a private conversation over tea between one of the APE representatives and Dr David Gower. The ethical lapse is the more serious since Dr Gower is seriously misrepresented as admitting "unenthusiastically" that he ascribed genetic defects to the fall and that sexual communication by pheromones has to do with "lustful temptations". These serious misrepresentations of a senior university academic are, to say the least, deplorable.

What your correspondents fail to grasp is that creationism is an holistic philosophy which seeks to understand the scientific enterprise within a biblical and theological framework. In pursuing this concept, and rejecting the arid materialism which underlies so much modern thinking, we are simply returning to the paradigm espoused by such as Newton, Boyle, Kelvin, Faraday and Maxwell, who among others laid the foundations of science as we know it.

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Riposte on CERN

SIR — Victor F. Weisskopf and J. H. Mulvey (*Nature* 8 October, pp.599-600) make various criticisms of our study of CERN, the European laboratory for particle physics. The criticisms are unfortunately misplaced since they relate to a brief *Nature* article (6 September, p.4) rather than to the detailed findings recently published in two parts in *Research Policy*.

First, we do not base our "main conclusions on a selection of so-called 'crucial' experiments" — we actually devote as much attention to evaluating "incremental" contributions to science. Indeed, the very first conclusion listed in the second of our papers is that since 1970, "the overall record of the CERN machines taken together has been better than that of the accelerators at any other laboratory in the world when judged in terms of experiments producing precise measurements". Nowhere do we describe such work as "dull physics".

Second, Mulvey's assertion that there is no evidence to support our conclusion that the multinational nature of CERN has previously led to problems in research management is wholly inaccurate. Even the most cursory reading of our papers would reveal that our evidence comes from interviews with more than 180 high-energy physicists, many of whom cited this particular problem as important.

Third, it is gross oversimplification by Mulvey to suggest that the greater success of the United States in terms of important discoveries was due to a single machine. The Brookhaven Alternating Gradient Synchrotron had a far better record than the very similar CERN Proton Synchrotron, as did Fermilab compared with the CERN Super Proton Synchrotron (SPS), while the CERN Intersecting Storage Rings "missed" several major discoveries in their energy range.

Fourth, the successes of Stanford with the SPEAR collider were not entirely unpredictable. Among other factors, they can be attributed partly to the fact that the machine opened up a new energy range (unlike the CERN SPS which began operating four years after an identical energy US machine), and partly to its users, who already boasted an impressive track record in exploiting the earlier Stanford linear accelerator.

Lastly, one of our papers devotes considerable attention to identifying the factors that have structured the performance of CERN accelerators. It is therefore incorrect to say that we offer no explanation for the improvement over recent years.

BEN MARTIN

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Irish students

SIR — We were surprised by your statement (18 October p.592) under the heading "Irish disunity" to the effect that "Roman Catholic students from Ulster tend to seek higher education south of the border when (*sic*) they are welcomed as if Irish".

This statement is patently false and could be unwittingly damaging. Although the Queen's University of Belfast, being completely non-sectarian, does not keep statistics of students' religions, it is common knowledge that the proportion of Catholic students at Queen's easily exceeds the proportion of Catholics in the population of Northern Ireland as a whole. The several thousands of Catholic students at Queen's alone, apart altogether from those in the non-sectarian University of Ulster, constitute a group at least four times larger than the total number of all Northern Ireland students who attend universities in the Irish Republic.

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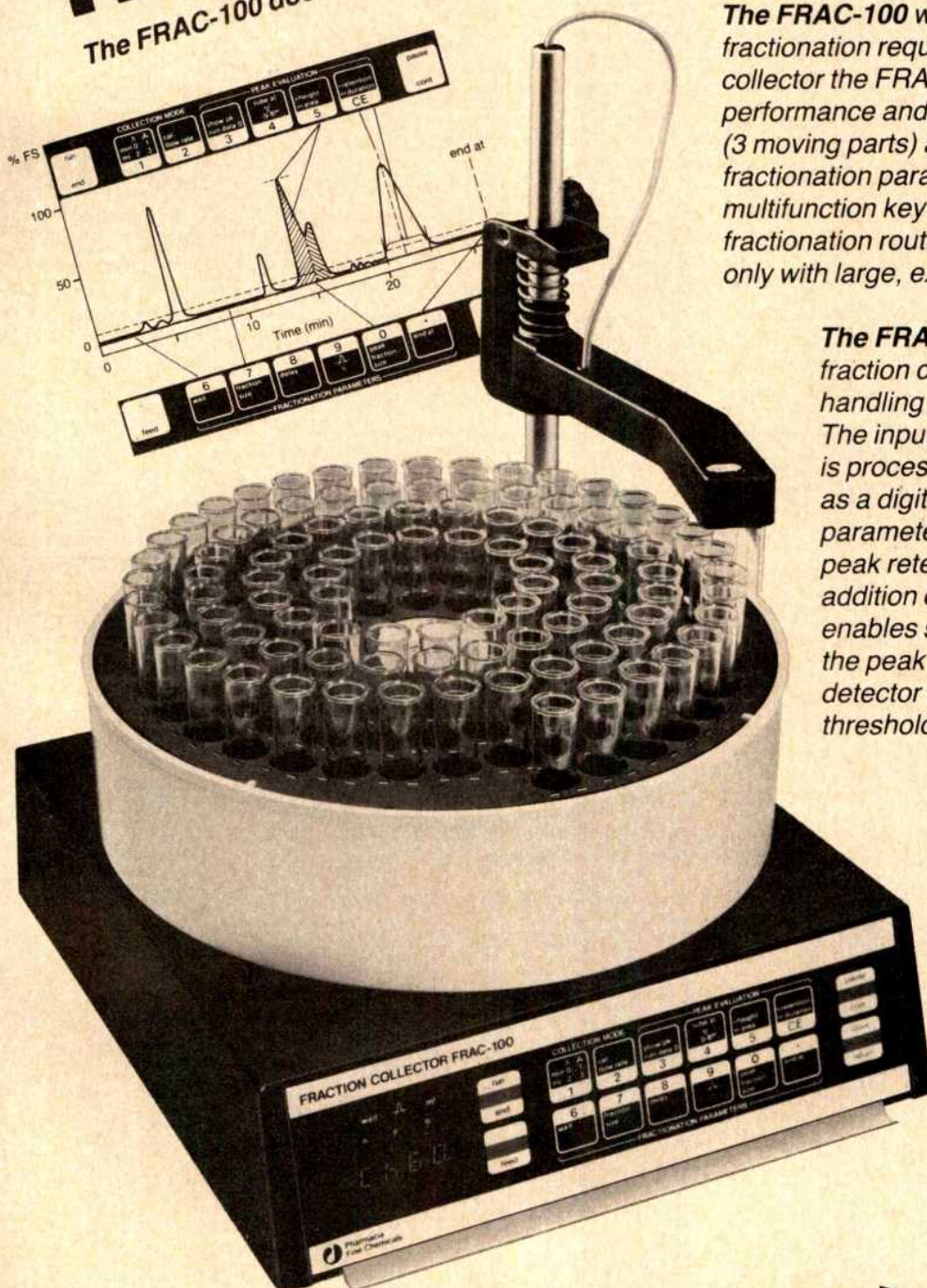
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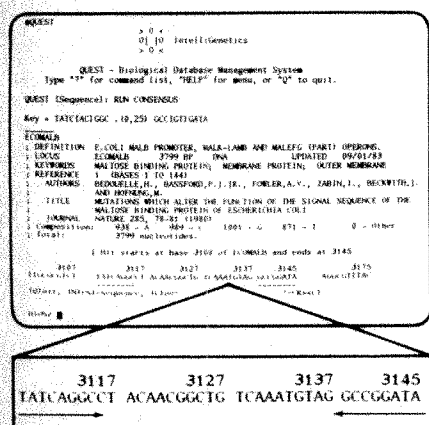
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Sequence Pattern Recognition Program Instrumental in the Discovery of a Unique Sequence Pattern in the Procaryotic Genome

SUMMARY:

QUEST, a sequence pattern recognition program developed by IntelliGenetics, allowed scientists at the Institute Pasteur to rapidly search all available nucleic acid databases for characteristic nucleic acid sequences initially observed in *E. Coli*. The scientists were able to discover the location of 67 members of a family of dispersed repetitive palindromic DNA sequences unique to the procaryotic genome.



These palindromic sequences could form stem and loop structures in RNA or be important recognition sites in DNA. The stem is composed of eight remarkably conserved base pairs separated by an intervening region that varies widely in length and sequence.

METHODS:

QUEST's sophisticated query formatting capabilities required only 38 sequence keys to represent over 2.3×10^{17} possible ambiguous sequences. These simplified keys were essential to the rapid search of the entire NIH GenBank™ and EMBL DNA sequence databases. For example, the pattern for the palindromic repeat "TATC[AC]GGC N{0, 25} GCC[GT]GATA" represents over 6×10^{15} possible different sequences. Additional levels of complexity and ambiguity may be easily represented in the QUEST searching patterns.

QUEST is based on a rapid pattern searching algorithm. Any pattern of either nucleic acid or amino acid sequence can be simply expressed regardless of the naturally occurring degree of ambiguity.

RESULTS:

Using the 38 QUEST keys, it was discovered that a characteristic 8 base pair inverted repeat (IR) sequence was found predominantly in procaryotic genomes. These palindromic IR's are present at least several hundred times in the genomes of *E. Coli* and other bacteria. These IR's are found primarily between genes and occur either singly or in clusters.*

CONCLUSION:

These inverted repeat regions, characteristic of procaryotes, support the cassette model of genomic evolution, and furthermore suggest a specific role for these sequences in genetic regulation in procaryotes.

QUEST is only one of a complete set of integrated computer resources developed by IntelliGenetics. Other programs which can help you in your research are:

CLONER Simulation and design of recombinant DNA experiments

MAP Determination of restriction maps from enzymatic digests

GENED Simplified entry of nucleic acid and amino acid sequence data

GEL Management of large scale DNA sequencing projects

SEQ/PEP Nucleic acid and amino acid sequence analyses and comparisons

IFIND High-speed nucleic or amino acid sequence homology searches

NIH, EMBL, NBRF On-line, up-to-date, annotated library of nucleic acid and amino sequences

IntelliGenetics is currently supporting over 500 molecular biologists worldwide with a complete set of programs, all fully compatible with the NIH GenBank™, EMBL nucleic acid databases and the NBRF protein sequence database. On-line support, training, consulting and documentation is provided for all products. IntelliGenetics' computer tools are available through a timesharing system, on the 32-bit supermicro BION graphic display workstation, or by software license for multi-user computer systems.

IntelliGenetics' QUEST and IFIND pattern recognition programs can now help you obtain results and reach new conclusions more rapidly than ever before.

To find out more about these powerful computer resources, call IntelliGenetics at (415) 328-4870, or send the coupon to: IntelliGenetics, 124 University Avenue, Palo Alto, California, 94301.



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*Accepted for publication in the European Molecular Biology Organization Journal, Vol 6, Number 3 (1984).

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Mind, body and intelligence

This year's BBC Reith lectures are arresting and provocative but also representative of the kind of argument that gives philosophers a poor reputation in research laboratories.

CAN machines think? And if so, in what sense? Those are two of the simple questions with which Professor John Searle of the University of California has been starting the British Broadcasting Corporation (BBC)'s audiences in the past few weeks. Professor Searle is this year's Reith lecturer, which means that he is one of a long line of distinguished scholars licensed to talk at British audiences for half an hour on each of six consecutive weeks. To judge from the first four of these occasions, Professor Searle is an iconoclast in the literal sense, one concerned with the destruction of idols. And there is no reason to think the worse of him on that account. But on this occasion he may have chosen too many idols, in too breathless a rush of broadcasting time, to satisfy the intellectual curiosity of his listeners.

Searle's general title is arresting — *Minds, brains and science*. His announced objective is to explore the relationship between the concepts of mind and body as illuminated by what has been learned of neurophysiology in recent years with the particular intention of trying to make sense of concepts such as free will, or "intentionality" as he puts it. And certainly it is an important question to answer, how to reconcile the behaviourists' view of people as predetermined exemplars of the laws of mechanics with the illusion we all share that in some sense we are in charge of our own destiny, or of events around us. Or, more accurately, where is the line to be drawn between the domains of conditioned and voluntary behaviour? And how fuzzy is that line?

We shall have to wait to see how Searle comes out on these questions. One striking measure of his success so far, however, is that he has made some of the subsidiary questions with which he has dealt into cocktail-party conversation. Is it, for example, true, as Searle asserted in the first of his lectures, that there is no serious problem about the distinction between mind and body? Body means a collection of neurones and mind means the qualities of consciousness, intentionality and so on that we attribute to ourselves. Searle's answer is that neurones are to be likened to the atoms that constitute physical objects, and that mind is the consequence of putting them together as they are in people, in every way the analogue of the external shape of the object concerned.

All this is unexceptional, even if it does not say much about the nature of the attri-

butes of the macroscopic representation, the nature of consciousness, for example. No doubt even philosophers will think Searle's answer a little over-simple, but it is no less arresting on that account. The circumstance that it leaves the question of how consciousness springs from the human nervous system to be answered by the neurophysiologists is neither surprising nor unwelcome, for the question is interesting and important.

The more serious difficulty with Searle's iconoclasm so far is that he seems to have chosen for attack some idols that are conspicuous by being insubstantial, which is where the question arises of whether machines can think. In a nutshell, Searle has been tempted into the sport of making fun of the community of computer-people who believe their creations are capable of artificial intelligence, or AI as they call it. As everybody knows, it is an easy sport, like shooting sitting ducks. It is also a misguided sport, certain with a British audience to evoke smug declarations that it has been clear all along that artificial intelligence is unreal.

Searle has made his point by means of a memorable example, one much talked about as irrefutable. Put a man in an empty room but provide him with a set of rules for combining Chinese ideograms together in ways that make sense (to Chinese speakers). Then imagine that a number of Chinese speakers outside the room are able to enter bundles of ideograms which the man must combine together according to the rules provided. To those outside the room, the man may well (when he is well practised at his task) be performing like an intelligent machine. But to the man, the process will be devoid of meaning. In this setting, Searle says, the system of man/room is exactly equivalent to a digital computer. If the people outside have omitted some essential rules, the system will be an unintelligent computer system.

The analogy is good because it is arresting and because it is also susceptible to further analysis. The difference between the man in the Chinese room and an intelligent human being, Searle says, is that the former has been provided merely with the formal syntax of the language with which he is working — the rules for telling how symbols should be combined — but that human beings have learned to attach meaning to the symbols. Semantics are an essential part of the intelligent manipulation of language.

Careful listeners to what Searle has been saying will recognize some important qualifications of what appears to be his case, that machines cannot think in the true sense of the word. First, he says, his conclusion applies to digital computers, not all machines; an artefact that exactly simulates a human being would be able to think well enough. Second, he acknowledges that the Chinese room is devoid of senses for inferring meaning from the outside world, so that it is bound to be semantically bereft. Unfortunately, having launched his commentary on the problem of the Chinese room by teasing the prophets of artificial intelligence for their frequent over-optimistic statements of the potential of their machines, he will have left his listeners with the impression that no machine can think.

Many of Searle's listeners will have been reminded of other such essays, perhaps that eleven years ago in which Sir James Lighthill, on a commission from the Science Research Council, concluded that the claims then being made (chiefly at the University of Edinburgh) on behalf of artificial intelligence were either schemes for making machine computation capable of more complicated tasks or schemes for the further automation of mechanical process. There were no proposals, said Lighthill, for bridging the gap — whereupon the research council turned its stoniest face towards grant-proposals in this field, setting back an interesting area of research for the best part of a decade.

Searle's distinction between syntax and semantics is helpful as far as it goes, but that, unfortunately, is not very far. It creates but does not bridge a gap. The snide riposte to the implied jibe that the Chinese room is necessarily stupid is to ask what would happen if the man inside were smart enough to figure out what all those ideograms really meant. The truth is that there is a gap, but one that is already narrower than when the Lighthill report appeared eleven years ago. And the purpose in seeking to fill the gap is not to replace people by machines (because, cost apart, people are more adaptive to changing circumstances than machines) but to understand better how people function (and, perhaps, to improve the automation of processes as a by-product). To fail to acknowledge this is what gives philosophers a bad name with people at the bench. Let us hope that Searle does better when he comes to free will. **John Maddox**

Burkitt's lymphoma

Clues to the role of malaria

from M.A. Epstein

STEADILY accumulating evidence that Epstein-Barr (EB) virus plays a crucial role in the chain of events which causes Burkitt's lymphoma in endemic zones culminated in the compelling findings of the World Health Organization's prospective study in Uganda¹. The virus seems to play its part by infecting and thereby 'immortalizing' a pool of B lymphocytes. Their continuous cell divisions facilitate any one of three characteristic chromosomal translocations that can activate the *c-myc* oncogene². However, since EB virus is ubiquitous whereas Burkitt's lymphoma has a high incidence only in remarkably restricted areas, it has long been recognized that a co-factor must determine the geographical distribution of the endemic tumours. Already fifteen years ago Denis Burkitt suggested that hyperendemic malaria was the co-factor and collected much supporting epidemiological evidence³. But possible mechanisms for its mode of action have remained obscure. On page 449 of this issue Whittle *et al.*⁴ provide the first clues.

Like all other herpes viruses, EB virus gives rise to a life-long infection to which the healthy carrier develops specific immunological responses. These are excellent at damping down the virus but never enough to eliminate it from the, as yet unidentified, immunologically-privileged site in which it persists. Thus, a delicate balance evolves. On the host side, the balance depends both on virus-neutralizing antibodies, which restrict the spread of the agent from one target cell to another, and on a full cellular immunological response, which is capable of destroying infected cells because they display viral antigens.

During primary EB virus infection, natural killer cells and other non-specific T cells may be of immediate importance. But thereafter, specific cytotoxic T lymphocytes (which are HLA-restricted) play a central role in maintaining life-long surveillance of the viral carrier state⁵. The very great importance of this surveillance is shown by the dire consequences when it fails: genetic defects lead to death from uncontrolled polyclonal proliferation of EB virus-immortalized cells, and widespread virus replication follows primary infection. Therapeutic immunosuppression in organ graft recipients also carries a considerable risk of malignant lymphoma⁶.

Although it has been known in general terms that malaria has profound effects on immune reactivity⁷, Whittle *et al.*⁴ now clearly demonstrate that the control of EB virus-specific T lymphocytes is dramatically impaired during acute malarial infection by *Plasmodium falciparum*. Interestingly, this impairment resembles

that of immunosuppressed renal allograft patients⁸. Moreover, while looking for changes in lymphocyte numbers that might explain the phenomenon, Whittle and colleagues have recorded further intriguing observations. Not only are T cell numbers decreased and B cells numbers increased, as might be expected, but the percentage of T-helper (T4) cells is reduced in relation to T-suppressor (T8) cells, giving a low T4/T8 ratio. It is not clear how malaria brings about these changes but the loss of T4 cells is not due to cytotoxic antibodies present in the acute phase serum.

A reduced T4/T8 ratio is one of the key hallmarks of acquired immune deficiency syndrome (AIDS), in which T4 lymphocytes are infected and destroyed by the retrovirus known as LAV⁹ or HTLV III¹⁰. Whittle *et al.* make the interesting speculation that a latent retrovirus of this group, after activation by malaria, might similarly account for the depressed T4/T8 ratios in their malaria patients.

However the damage to specific T-lymphocyte control in malaria arises, it is not difficult to envisage that in the hyperendemic disease, with its repeated attacks throughout each and every year, such damage will favour the unrestrained growth of EB virus-carrying B cells in a substantial number of individuals. A firm

immunological explanation for the geographical coincidence of endemic Burkitt's lymphoma with hyperendemic malaria has thus been provided by experiments of Whittle *et al.*, even though the exact cause has not yet been identified.

Other loose ends remain to be tied up. Even where EB virus and hyperendemic malaria coexist in populations, the results of the prospective study in Uganda suggest that certain individuals are at special risk. Evidence for a genetic predisposition has not been forthcoming and should perhaps be more rigorously sought for. It has also been suggested that infection by the virus very early in life might be a predisposing factor¹¹; this too requires clarification. Alternatively, some special sequential relationship between the primary viral infection and the malarial infection could be important either in determining damage to the EB virus-specific T cells, or in providing unusual target B cells, which more readily escape from the damaged control mechanisms. □

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11. De-Thé, G. *Lancet* **i**, 335 (1977).

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Mathematics

Five bodies to infinity

from Ian Stewart

TYCHO Brahe's observations of planetary motion led Johannes Kepler to the idea that planets orbit the Sun in ellipses. Isaac Newton showed that this would follow from an inverse square law of attraction. A fully rigorous and complete treatment of the problem of the motion of two bodies under Newtonian gravitation was given by Leonhard Euler in 1744. The extension to more than two bodies has proved considerably less tractable. While excellent approximate methods exist (and are widely used in astronomy and space exploration), general theoretical insights seem hard to come by. The problem is deep and difficult, the phenomena are complex. Just *how* complex is shown by J. Gerver in the *Journal of Differential Equations* **52**, 76 (1984).

Gerver gives a heuristic but convincing argument for the existence of configurations of five point masses which, under their mutual Newtonian gravitational attraction, fling themselves out to infinity in a finite time. This relates to an important theoretical issue: the possible occurrence of

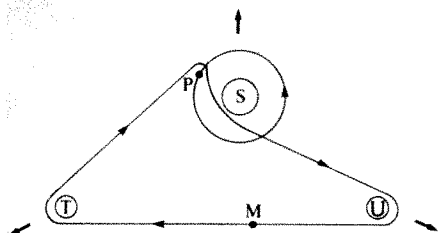
singularities, that is events beyond which the motion cannot be continued within the mathematical model. In 1897, Paul Painlevé proved that for three bodies, every singularity must be a collision. Collision singularities are fairly well behaved: in fact if only two bodies collide at a given instant, then the motion can be 'desingularized' by assuming elastic collisions. But Painlevé also suggested that with more than three bodies there might exist much nastier types of singularity such as bodies escaping to infinity in a finite time, or oscillating ever more violently.

In 1970, H.J. Sperling proved that in order to have a singularity without collisions, at least one body must escape to infinity; and seven years later D. G. Saari showed that one of the escapees would have to oscillate wildly. He also showed that, in the case of four bodies, they would have to approach a straight line arrangement as time increased. In 1974, J. Mather and R. McGehee, studying an idealization, showed that escape to infinity in finite time

could occur for four point particles constrained to lie on a line and colliding elastically. The process involves an infinite number of energy transfers, happening ever more rapidly: conservation of energy does not rule out such escapes.

Gerver argues that in the (planar or three-dimensional) five-body problem, non-collision singularities should exist. The proposed mechanism is ingenious. Imagine three massive 'stars' S, T and U represented by point masses. Make S slightly heavier than T and U. Arrange all three stars in a triangular constellation with an obtuse angle at S, and start them out in such a way that the triangle expands at a uniform rate while maintaining its shape. Add a tiny 'meteorite' M, which orbits round the outside of all three, approaching them very closely (see figure).

As M passes each star it undergoes the same 'slingshot' effect that was used for the Voyager spacecraft journeys through the



Solar System. As a result, M gains a small amount of kinetic energy from star S, which it can then share with T and U to increase their speeds (and its own). If only there were a way also to increase the energy of S, it would be possible to make the triangle expand at an ever increasing rate, disappearing to infinity in a finite time.

Of course, conservation of energy forbids this — but there is a way out. Place a fifth 'planet', P, in a roughly circular orbit round S. Now make M gain its energy at the expense of P and transfer some of it to S via a 'zig-zag' slingshot path. Otherwise proceed as before. Now, as time increases, the triangular constellation expands ever faster but does not change its shape, M moves ever more rapidly in its triangular orbit, and the orbit of P descends ever closer to S. As a result, all five bodies escape to infinity in a finite time.

This scenario requires a very delicate balancing act. For example, planet P must be in a suitable position for the zig-zag slingshot not just at the first pass, but at every pass; its orbit must stay roughly circular; and the triangle of stars must stay stable. Furthermore, the energy transfers must be handled in such a way that the increase in the speed of the meteorite as it traverses the constellation is greater than the rate at which the constellation grows, so that the orbital period of the meteorite shrinks. Gerver argues plausibly (but heuristically) for the existence of a set of initial conditions that keeps all of these problems under control. He adds that a calculation "nearly one hundred pages long" give a rigorous proof that the 'Great Escape' does occur in an analogous model

problem, in which some particles are massless and the meteorite undergoes elastic collisions instead of slingshots. The full result might be proved in "hundreds of pages of calculations". A better approach might be to seek new methods to make the arguments more rigorous.

In the real Universe, of course, additional bodies would interfere with this deli-

cate scenario, and relativistic effects would change the results. But Gerver's idea is a bold and imaginative thrust at the heart of a celebrated problem, and a challenge to the current mathematical techniques. Isaac Newton would have loved it. □

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Radiocarbon dating

New World colonized in Holocene

from Richard Burleigh

A PAPER by J.L. Bada *et al.* on page 442 of this issue describes the application of the new accelerator mass spectrometric (AMS) technique of radiocarbon dating to human skeletal remains from California. The dating of the remains to the Holocene is important for two reasons: first, it adds to the increasing body of evidence placing the human colonization of the New World in this period; and second, with much wider relevance, it provides a striking demonstration of the power of AMS.

The time of first arrival of humans in the New World has long been a matter for controversy. During the earlier decades of this century any claims for the antiquity of apparently early human skeletal remains discovered in the Americas were hotly contested, notably by the physical anthropologist Ales Hrdlička. At their most vehement, Hrdlička's views would not allow the presence of humans there for more than a few millennia, although eventually he was prepared to concede a possible time of arrival of 10,000–15,000 yr ago. The most widely accepted view today is that human groups first entered North America some 12,000–13,000 yr ago after the ice had begun to retreat at the end of the last glaciation. They most probably came across a land bridge from Asia (Beringia) rather than by a coastal (sea) route. On this model, if human remains very much older than about 12,000 yr before the present (BP) were to be found in the Americas, it would suggest that entry had occurred during the previous interglacial, some 70,000–100,000 yr BP; there is no fossil or lithic (artefact) evidence in support of this. In other words, the first people probably arrived in the Holocene (postglacial) rather than during the Pleistocene. Conventional radiocarbon dating over the past 25 years has contributed support for this time scale for the appearance of humans in North America and for their subsequent very rapid spread southwards.

Racemization dates of up to 50,000 or even 70,000 yr BP, obtained by Bada and his colleagues at the Scripps Institution in the mid-1970s for amino acids extracted from human skeletal remains from California (notably those from Del Mar and Sunnyvale), were therefore in immediate conflict with accepted views of

the antiquity of the human population of North America deduced from radiocarbon evidence. On the other hand, although these dates seemed incredible they could not be lightly dismissed. The measurements were carefully made, apparently independently 'calibrated' by a radiocarbon date of 17,000 yr (UCLA-1233A) for a human skull from Laguna Beach, California (itself an unexpectedly early date), and, above all, were direct analyses of the skeletal remains themselves.

The opportunity to test the validity of these results comes with the successful development over the past seven years of the AMS technique of radiocarbon dating (Hedges, R.E.M. & Gowlett, J.A.J. *Nature* 308, 403; 1984) which requires only a few milligrams of sample, in contrast to conventional radiocarbon techniques, where gram quantities are needed. In their current work, Bada *et al.* have used the AMS technique to date aliquots of the original amino acid extracts used to obtain the racemization dates. Their new results (which are well-supported by some conventional ^{14}C measurements of related acid-insoluble extracts) clearly show that all the remains, including the Laguna skull, are of Holocene age. This agrees with the date reported by Stafford *et al.* (*Nature* 308, 446; 1984), of less than 4,000 yr BP obtained using AMS for a human burial from Yuha, California, that had previously been assigned a racemization age of some 24,000 yr. Using these new results, the cranium of Los Angeles Man, previously dated by the conventional radiocarbon technique to greater than 23,600 yr BP (UCLA-1430; Berger, R. *World Archeol.* 7, 174; 1975), is most probably also of Holocene age. These new results remove a puzzling anomaly that was in conflict not only with a model built very carefully over a long period from many independent (archaeological, geological, environmental and climatic) lines of evidence, but also with the observation that these human skeletal remains are essentially modern in appearance and show few if any of the traits that might be expected in the representatives of earlier populations. □

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Geology

Death of an ancient ocean

from Peter Gambles

THE Caledonide Orogen is the name given to the mountain belt, now torn apart by the modern Atlantic ocean, that was formed by the collision of the continental plates of North America, Africa and Baltica between about 550 and 350 million years ago. Stretching from Spitzbergen and Greenland in the north, to the Mauritanides and the Gulf of Mexico, it is one of the longest such belts in the world. The broad outline of the plate tectonic movements that formed the mountains has been known for some time, but their reconstruction still resembles a child's drawing with straight-edged continents closing over a smooth ocean floor. A glance at any globe will show that this is not the real world. Oceans are dotted with mid-ocean ridges, volcanic arcs and islands of both oceanic and continental crust; moreover, continental margins are far from straight. The exciting challenge now was made clear at the symposium on 'Evolution of the Caledonide-Appalachian Orogen', University of Glasgow,

3-8 September 1984. It is to integrate the wealth of data from traditionally distinct disciplines within earth sciences into a more realistic model of this major episode in the Earth's history.

The fashionable concept of suspect or allochthonous terranes is part of the new approach to plate tectonics. In essence, such a terrane is a sizeable chunk of continental crust, the geology of which is incompatible with that of adjacent areas. The Western Cordillera in the United States and Canada is now thought to comprise a multitude of such microcontinents. Many of these have moved hundreds of kilometres, largely parallel to the continental margin. By contrast, some of the terranes in the Appalachians have been transported from one continent to another across the oceans.

To establish the links between the Appalachian terranes and those now on the opposite side of the Atlantic, geologists must rely on the traditional methods of

comparative stratigraphy, using as a standard the Newfoundland portion of the orogen — the Humber, Dunnage, Gander and Avalon zones. Of these, the Avalon is probably the most significant in terms of the evolution of the mountain belt, and has proved useful, for example, in examining the very earliest development of the Caledonide Orogen. During the latest Precambrian (700–580 Myr), the eastern margin of North America underwent a large degree of crustal extension (D.W. Rankin, USGS, Reston). This is indicated in the geological record by the presence of basaltic dykes, forming up to 50 per cent of the exposed rocks, and by the extrusion of continental tholeiitic and alkali basalts. However, contemporaneous volcanism in Avalon was calc-alkaline, and so not related to rifting, suggesting that Avalon was not part of North America. Moreover, the distribution of fauna, especially trilobites (L.R.M. Cocks, British Museum and W.S. McKerrow, University of Oxford), favours closer links of Avalon with Gondwana (a supercontinent comprising today's southern continents including Africa) than with America during the early Cambrian (570 Myr). To the north of the continental fragment lay a number of ridges and basins such as the Irish Sea

THE evolution of the mountainous Caledonides of Scandinavia and Britain, the Appalachians of North America and Canada and the Mauritanides of North Africa has been the subject of a 10-year programme of geological research entitled 'The Caledonide Orogen'. Organized jointly by Unesco and the International Union of Geological Sciences, it has been led by a group of Norwegian geoscientists. Together over 200 specialists from countries bordering the present north Atlantic have carried out nationally financed research projects and exchanged results at a series of symposia held in conjunction with field studies.

The final symposium of the project was one with a difference in that the entire orogen was studied in terms of four time slices rather than in the more conventional divisions into subject specializations. Some of the coordinators of these sessions had clearly done their homework, while others, for one reason or another, were forced to rely on lecturers who had only a vague knowledge of what the Caledonide Orogen was and even less knowledge of its present day geography.

The scene was set by the geophysicists, whose models of the earth, based partly on data beyond the reach of the field geologist, must be tested against the field evidence. The two approaches sometimes conflict. For example, fossils and sediments may indicate a warm climate, while the geomagnetic palaeopole position suggests the contrary. In my view the evidence for an equatorially placed American plate during the Ordovician

period and for a high latitude position for Baltoscandia (~60°S) during at least the first half of the period, is overwhelming. The majority of geologists are prepared to accept this. What is not certain, however, is the relative positions of both plates and that of the African. Did they meet along a so-called triple junction, and were Africa and Baltoscandia separated by the so-called Tornquist Sea?

Meanwhile the palaeontologists continue to provide dates (with an accuracy of one million years in some instances) and information which both startles and irritates. Yet they cannot be disregarded, even when they happen not to fit some of the many, and often conflicting, dates produced at vast expense by radiometric methods. I was amazed at how uncritically some of the radiometric dates were used to fit the geological models. A fossil cannot be reset nor can it be destroyed (unless lost). It can be wrongly interpreted but it is a firm piece of tangible evidence. The Caledonide Orogen project has done more than any other to encourage palaeontologists to provide answers to questions posed by field geologists rather than other palaeontologists. Their diligent collecting has provided a wealth of material from the most unlikely rocks; shelly fossils have even been found in volcanic ashes derived from oceanic islands along the length of the belt.

Perhaps the most successful part of the whole project, and certainly the most exciting session of the symposium, concerned geological maps. From the inception of the project, specialist study groups were set up with the specific goal of

producing a series of 1:1 million scale geological maps of the Caledonide-Appalachian Orogen. In addition to the more usual maps of geological structures, the distribution of volcanic and plutonic rocks, and the relationships between sedimentary rock types and faunas, there were a number of more adventurous projects. These covered the timing of deformation and age of metamorphism in various parts of the orogen, and the relationship between basement rocks and their cover, topics almost designed to be controversial. From the start, a standard time scale was agreed on, as were symbols and colours. The end result is that British and Irish geologists cooperated to provide three handsome new maps, on sale at the symposium, and Norwegian and Swedish geologists jointly produced a map with boundaries that actually coincide where they cross the state border. Regrettably, a number of the thematic maps are still provisional and there is doubt as to whether funds to publish them will be forthcoming.

When the final speech of the symposium declared the end of the project, those of us who thought it an enjoyable and worthwhile experience have the prospect of another to look forward to. 'Suspect terranes', as it may be called, will be considered by the board of the International Union of Geological Societies in 1985. □

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landmass and the Leinster-Manx and Welsh Basins, separated from America by a major ocean — the Iapetus. The history of the Caledonide Orogen in this region thus appears to be concerned with the closure of the Iapetus Ocean, but not, as previously thought, with its opening.

Between Avalon and Africa (Gondwana) was a narrow basin termed the Cadomian Ocean. Palaeomagnetic data suggests that during the late Cambrian–early Ordovician (520–480 Myr), Gondwana rotated about a pole in eastern Africa, thereby closing up this seaway (C.R. Scotese, University of Texas, Austin). This is in agreement with the sedimentological evidence for infilling of the basin by shallow water sandstones (F.L. Schwab, Washington and Lee University).

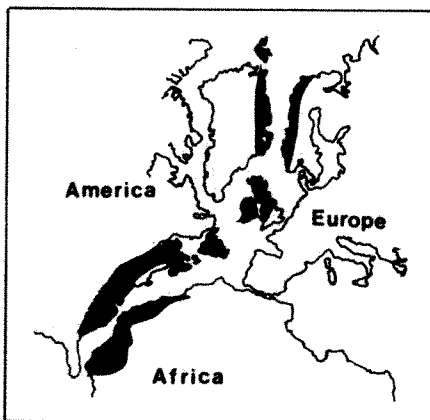
From the middle Ordovician (470 Myr) onwards, a number of fragments of island arcs, formed by subduction of the Iapetus Ocean under North America, were split off from the continent by inter-arc basins, which in turn were rapidly filled with sediments derived from volcanoes (C.J. Stillman, Trinity College, Dublin). All this material was variously subducted or obducted as it moved northwards towards the craton. Pieces of oceanic crust, such as the Bay of Islands ophiolite, were trapped during this pulse of tectonic movement but interestingly very little geochemically-normal open oceanic crust from Iapetus has been found. The rocks of the continental margin and Humber zone were deformed and metamorphosed during the middle Ordovician, but the absence of such effects from the Dunnage and Gander zones suggests that they were by then accreted to Avalon as it moved northwards, impacting with North America by the late Devonian (370 Myr) and opening the Theic Ocean behind it.

This style of plate tectonics — the shuffling of continental blocks or suspect terranes — can be traced from the southernmost parts of the Appalachians to northern Scotland. Across the North Sea, the picture is dramatically different. The Caledonides in Scandinavia are a classic example of large-scale thrust tectonics. The mountain chain is built up of four allochthonous units that are believed to have travelled considerable distances — a theory familiar to Alpine geologists but less readily accepted by the British.

Cocks presented palaeontological evidence indicating that at least the upper two nappes in Scandinavia were originally part of the North American plate. Ophiolites are restricted to these two sheets which, in the manner typical of nappe stacks, originated furthest from their present position. All the nappes contain volcanic rocks, geochemical study of which (H. Furnes, University of Bergen) shows that the lowest three were involved in rifting and only the uppermost contains calc-alkaline sequences characteristic of an island arc. The Baltic coast of the Iapetus Ocean thus appears to have been a passive plate tec-

tonic margin, with the oceanic crust being subducted under North America or Greenland. The Scandinavian Caledonides are considerably deformed and metamorphosed, the emplacement of the nappes being one of the last tectonic events. R.D. Dallemeyer (University of Georgia) has used ^{39}Ar – ^{40}Ar dating of individual minerals (in itself a technical achievement) to demonstrate very neatly that in Scandinavia there were two distinct orogenic phases, termed the Finnmarkian (500–460 Myr) and the Scandian (420–400 Myr).

During the early Silurian, sheets of Iapetus sediments and crust were thus being thrust eastwards onto the Baltic craton. What of the opposite side of the ocean? In east Greenland, K–Ar ages (also an indicator of deformation and low grade metamorphism) of 445–410 Myr are recorded in the main fold belt (R.J. Tracey,



Distribution of the Caledonide-Appalachian-Mauritanide mountain chain (in black) before the opening of the present Atlantic.

Yale University) and it is now thought that much of the material in Greenland which underwent Caledonian metamorphism may in fact be allochthonous on Precambrian (Grenville) basement.

As McKerrow pointed out, the problem that then arises is how, during the Scandian orogeny, could nappes have been thrust eastwards on to the eastern margin of the Iapetus Ocean (Norway) at the same time as others were being forced westwards on to the western margin (Greenland)? His elegant solution is to split the subduction zone down which the crust of the Iapetus was disappearing into two, by means of an east–west oriented transform fault, across which the direction of subduction changes. A large amount of strike-slip motion is further involved, with the various parts of Spitzbergen being strung out on either side of the subduction zone dipping westwards under south Greenland. W.B. Harland (Cambridge University) argued that east Spitzbergen was positioned off east Greenland in the early Palaeozoic, while the geology of west Spitzbergen resembles that of north Greenland more closely. The fascinating possibility arises that Norway and Greenland swapped thrust sheets and that Spitzbergen was squeezed out northwards as the Iapetus closed.

So what separated the northern part of

the Caledonide Orogen from the southern part? The answer lies in the Tornquist line — one of the major ancient lineaments to have cut the crust of the continents since the Precambrian and which stretches from the Black Sea through Poland and Denmark to the North Sea.

Most Western geologists believe that there was a Tornquist Sea (or Rheic Ocean) which separated Baltica from Gondwanaland (including south Europe) until the latest Ordovician (440 Myr). Their belief is primarily based on the presence of two distinct faunal provinces which broke down only in the early Silurian. Unrefined palaeomagnetic data seem to suggest that the Tornquist Sea existed well into the Devonian (380 Myr). Polish geologists, however, find no evidence of deformation due to the closure of this putative seaway (B. Lewinski, Polish Geological Survey), though they have identified a major fault at depth from seismic studies, and recognize various phases of movement along the Tornquist line. Lewinski suggests that the faunal provinces can be accounted for by other barriers, such as climatic belts.

As can be seen from the Mediterranean, the closing stages of the collision of major plates are very complex. Some areas consolidate whilst small basins develop in others, perhaps with minor amounts of oceanic crust flooring them. J. Rodgers (Yale University) suggested that the ocean between Avalon and Africa still contained islands of 'Avalonia' after the main fragment was consolidated to North America. This is likely also to have been the case in the Theic Ocean and perhaps pieces of the European continental mosaic can be taken and placed in the Tornquist gap, thus eliminating the need for a true ocean. (The Tornquist line runs straight into the North Sea, which contains no normal oceanic crust. If the North Sea were to close, what evidence would remain of its existence in 400 million years time?)

The Caledonide Orogen was thus not simply the closure of the Iapetus Ocean. Rather, a picture emerges of a southern realm dotted with large fragments of continental blocks forming its margins, which were jostled about as Gondwana pushed relentlessly towards America. At the same time the northern arm of the Iapetus closed progressively from the north, thrusting great thicknesses of rocks onto the cratons on either side. The final locking of the three continents of Gondwana, North America and Baltica in the late Devonian (370 Myr) produced the supercontinent of Pangaea. But within a few tens of millions of years, far less than the lifespan of the orogen, Pangaea started to break up with renewed rifting in the Oslo and Rhine regions, leading to the opening of the Atlantic Ocean. In some ways, therefore, modern geology is but a continuation of the Caledonide Orogen, as the dance of the continents goes on. □

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Drosophila development

Smell taste and rhythm

from Michael Ashburner

ALREADY hurt by the news that, with respect to the development of its nervous system, *Drosophila* is but a small grasshopper¹, drosophologists assembled in Crete last summer* learnt that the central nervous system of their favorite fly may not be so different from their own.

M. Heisenberg (University of Würzburg), reviewed the evidence from his laboratory² that the mushroom body, a region of the brain of insects thought to be important for conditioned behaviour, is developmentally plastic. The number of mushroom body fibres normally increases in the first week of adult life. Extraordinarily, this increase does not occur if the flies are kept in a deprived environment, for example alone rather than with their fellows. Heisenberg's group have isolated mutations that effect the mushroom body. In one, the Kenyon cells — neurones intrinsic to the mushroom bodies — degenerate in female larvae. Although flies with mushroom body defects can smell, they have impaired olfactory conditioning. What is the significance of this? A clue may come from flies that are homozygous for mutations of the *tra-2* gene. These flies develop as phenotypic males even if they have two X chromosomes.

A temperature sensitive allele of *tra-2*, *tra-2^{ts}*, allows female development at 16°C but male development at 29°C. Strangely, however, if 16°C females are kept for six days at 29°C as adult flies, they begin to show male sexual behaviour³. It seems to be a reasonable hypothesis that the behavioural plasticity revealed by the experiment with *tra-2^{ts}* and the morphological plasticity seen in the mushroom bodies are related, suggesting a role of olfactory conditioning in the sexual behaviour of the flies.

Drosophila is a sugar-lover, with separate receptors for pyranose, furanose and trehalose on its feet and labellum (O. Siddiqui, Tata Institute, Bombay). Mutations that affect chemoreception may either influence the receptors themselves, such as *gust A* which eliminates the pyranose response, or act centrally. *Gust R* is an interesting member of the latter class, since it creates a fly that is quite indifferent to all sugars yet is abnormally attracted by salt. Siddiqui finds that the response of the sugar and salt receptors is normal and explains the phenotype as a result of central processing of inputs from the salt receptors as if they had originated from sugar receptors.

Many years ago R. Konopka, then at California Institute of Technology, ran a heroic hunt for X-linked mutations that

affect the circadian rhythm of eclosion of flies from the pupal case. Wild type flies eclose with a rhythm of 24 hours. Konopka found three mutations — one abolished all evidence of rhythm, one lengthened the period of the rhythm to 29 hours and one shortened it to 19 hours. All three were alleles of a single locus — called *per*, for *period*⁴. Both M. Young (Rockefeller University) and M. Robash with J. Hall (Brandeis University) have now cloned *per*. Dramatically, the Brandeis group find that a 0.9 kilobase transcript from the *per* locus is at least 10-fold more abundant at noon than at midnight, even in flies that are kept under constant dark conditions. Moreover the arrhythmic phenotype of the *per*⁰ mutation has been corrected with cloned *per* by germ-line transformation. Of course, we are still some way from understanding how the *per* gene product controls the circadian behaviour of flies — let alone its role in other rhythms⁵.

R. Wyman and J. Thomas (University of Yale) described the isolation of *Drosophila* mutants that cannot escape the fly swatter's swat. Two of their mutations disrupt the synapses between the giant fibre, which descends from the brain, and the jump motor neurone: *bendless* affects the presynaptic cell — the giant fibre's 'bending' branch, which should make the synapse, is simply missing; *passover* affects the postsynaptic cell — the central branch of the jump motor neurone passes right over the giant fibre without making a proper synapse⁶. Wyman speculates that the wild-type alleles of these genes encode molecules required for cellular recognition during neurogenesis. The wonder, of course, is that the mutant phenotype is so specific.

Growth and development of *Drosophila*, in common with all other insects, is coordinated by a steroid hormone, ecdysterone. Four genes that respond to ecdysterone have now been cloned. Two correspond to the well-known puffs of the larval salivary gland 74EF and 75B (D. Hogness, Stanford University); the other two code for proteins synthesized as an early response to ecdysterone by Kc tissue-culture cells (P. Cherbas, Harvard University). The former pair, called E74 and E75, are both gigantic. E74 has a 62 kb primary transcript, processed by the removal of at least six introns to a putative mRNA of 5.9 kb with a mere 0.9 kb open reading frame, and E75 has a primary transcript of at least 50 kb which is processed to 5 and 6 kb products. Following induction by ecdysterone, these RNAs accumulate and then, within 2–4 hours, decline in a way that closely parallels the behaviour of the salivary gland puffs.

E74, at least, does not appear to be tissue specific. One of the genes described by Cherbas can produce two proteins by means of alternative splicing of the gene transcript and has at least four other transcripts which show a high degree of tissue specificity. It is conceivable that there is a family of related ecdysteroid-induced proteins, differences in which may play a role in the specificity of response seen at the cellular level. Intriguingly Cherbas has evidence for symmetrical transcription from both strands of ecdysterone-inducible genes.

No Kolymbari meeting can be considered complete without discussion of heat shock and transposable elements. One chromosomal puff (93D) produced by heat-shocking *D. melanogaster* has long been an enigma, for all the evidence has suggested that it does not code for proteins. Moreover it alone is induced by benzamide⁷ and is also full of large ribonucleoprotein granules. At last 93D has been cloned by both B. Hovermann and E. Bautz (University of Heidelberg) and by M.L. Pardue (MIT). The region transcribed after heat shock is made up of tandemly repeated 280 bp units cut by the enzyme *TaqI* and little else. The homologous puff of *D. hydei* (48C) has a 115 bp repeat that shows no homology with that of 93D, despite the fact that the regions which flank the repeats are homologous in these species. On heat shock, the 93D puff accumulates a ribonucleoprotein-associated antigen known as P11. Bautz suggests that perhaps 93D is a refuge for mRNAs, protected by P11, during the time that their transcription is blocked by heat shock.

Applications of P element-mediated germ-line transformation of *Drosophila*⁸ include the rescue of a mutant phenotype with a cloned DNA fragment, thereby confirming the isolation of the mutation's wild-type allele. A dramatic extension of this method was discussed by V. Pirrota (EMBL, Heidelberg), who has rescued a mutation by transformation with a 40 kb fragment on a cosmid vector with P element ends. One of the surprises of the work with transformed *Drosophila* strains has been that most genes function more or less normally in development regardless of the position of their chromosomal integration. Some influence of position on expression can however be seen. For example, G. Richards (CNRS, Strasbourg) has an integrant of the salivary gland glue gene *Sgs-3* that is almost inactive despite (or because?) of its location within 0.8 kb of the resident *Sgs-3* gene.

Richards and others are using transformation to identify *cis*-acting sequences that are required for proper gene expression. Thus, the alcohol dehydrogenase gene is expressed in larval salivary glands after transformation with a construct of the gene and the up-stream noncoding sequence of a salivary gland glue gene (S. Beckendorf, University of California, Berkeley). However, there is also gene

* The fourth EMBO workshop on 'Molecular Biology and Development of *Drosophila*', Orthodox Academy of Kolymbari, Crete, 24–29 June 1984. The sessions on homeotic genes were reported in *Nature*, 20 September, p.214.

expression in the gut, so this is not simply a case of the effects of a tissue-specific enhancer.

The analysis of *cis*-acting elements required for the tissue and temporal specificity of gene expression is in its early days. Yet it would be safe to predict that it will be a topic that will dominate discussions at the next Kolymbari meeting, that will take place in 1986. □

Cell biology

Actin-binding protein evolution

from Thomas D. Pollard

READERS of *Nature* must find the seemingly endless proliferation of actin-binding proteins a bit overwhelming. They might well ask when will it end and how does the paper on page 424 by Maruta *et al.*¹, describing the possible evolutionary origin of *Physarum* capping proteins, fit into the story?

Let us consider first where the field stands and where it might be headed. With some rare exceptions, actin is present in the cytoplasm at concentrations up to several hundred micromolar, higher than that of any other protein. Such concentrations of purified actin would polymerize very rapidly, leaving less than one per cent in monomeric form at physiological salt concentrations. In cells, only part of the actin is polymerized and a repertoire of regulatory proteins appear to govern the time, place and extent of polymerization and to organize the filaments into higher-order structures (see Table). Specific regulatory proteins probably exist for each step in the assembly process, including nucleation, growth at the two ends of the polymer and fragmentation of polymers.

Although more than 60 such actin-binding proteins have been described from various tissues, each passing month makes it clearer that they will eventually all fit into a limited number of families. (The problem, as one wag has put it, is that scientists would rather use each others' toothbrushes than their nomenclature, hence there is usually more than one name for similar proteins.) More importantly, I now feel it is safe to predict that representatives of at least most, and perhaps all, of these families are present in all non-muscle cells. Consequently, information about a new actin-binding protein isolated from *Acanthamoeba*, *Dictyostelium* or *Physarum* (the three favourite 'primitive' cells for such studies) is likely to be informative about homologous proteins in vertebrate cells.

With this in mind, what have we learned from the new work of Maruta *et al.* on *Physarum* capping proteins? The proteins in question are part of a family of Ca^{2+} -sensitive proteins that can both fragment actin filaments and block their 'barbed' (fast-growing) end. (There are three other

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families of these proteins that bind to the ends of actin filaments; see the Table.) Intriguingly, Maruta *et al.* have found that three actin filament-capping proteins — fragmin², Cap 42(a) and Cap 42(b)³ — share some features with actin itself: all four proteins bind ATP; there is partial cross-reactivity among antibodies to the four proteins; and pairs of the proteins have similar peptide maps. There are

capping protein has retained the sites used by actin to bind to the end of the filament but has lost (or modified) the sites used by actin to bind the next subunit in the polymer. Such a modified actin would be ideal for capping the end of the polymer.

Have other actin-binding proteins evolved by modification of an actin gene? Although not conclusive, most evidence suggests that they are not related to actin. For example, the sequence information available on profilin⁴ and gelsolin⁵ reveal no similarity to actin. Fingerprints of profilin, actophorin, capping protein and actin from *Acanthamoeba*⁶ are clearly different. Finally, I know of no other case where an antibody to actin-binding proteins cross-reacts with actin; in fact, with the exception of some antibodies to spectrin⁷, most antibodies to actin-binding proteins show no reactivity with proteins outside their own class.

The organization of the actin system will be understood only when the catalogue of components is completed and the mechanism of action of each protein has been elucidated in detail. Current work suggests that these mechanisms will be

Distribution of actin-binding proteins				Other lower eukaryotes		
Class	Families	Subunit MW	Protozoa	eukaryotes	Vertebrates	
Bind actin monomers	Profilin	12–15K	+	+	+	
	Depactin/actophorin	16–20K	+	+	+	
Bind end of actin filaments	Capping protein	29K + 31K	+	+	+	
	Fragmin/severin	40–45K	0	+	+	
	Accumbatin	65K	0	0	+	
	Gelsolin/villin	90–95K	0	0	+	
Bind along actin filaments	Tropomyosin	30–40K	0	0	+	
Cross-link actin filaments to each other	Gelactins	23–38K	+	0	0	
	Fascin/fimbrin	55–70K	0	+	+	
	α -Actinin	90–100K	+	+	+	
	Actin-binding protein/filamin	250K	0	0	+	
Cross-link actin filaments to other structures	Brush border	110K	0	0	+	
	Spectrin	220–260K	+	+	+	
	Microtubule-associated protein 2	~260K	0	0	+	
Myosins	Myosin/myosin-II	175–220K	+	+	+	
	Myosin-I	125–130K	+	0	0	

Actin-binding proteins are grouped into classes by their established properties and subdivided into families according to physical properties and presumed mechanisms of action. This classification is arbitrary and will probably be modified as more data become available. +, the protein has been identified in these cells; 0, the protein has not yet been identified in these cells (or rarely, for example in the case of tropomyosin in protozoa, the protein has been sought but not found). MW, molecular weight.

equally clear differences: actin and Cap 42(b) bind to DNase I but fragmin and Cap 42(a) do not; there are kinases specific for subsets of these proteins; and nucleotide-binding properties of the proteins differ. The main difference, of course, is that actin polymerizes whereas the capping proteins interfere with this process by blocking the fast-growing end.

The fascinating suggestion of this work is that at least one class of actin-binding proteins has evolved by duplication and modification of the actin gene itself. This idea will have to be substantiated by examining the primary structure of the proteins and their genes but, if true, argues for a clever economy in the evolutionary processes. Perhaps the actin gene has been modified selectively, so that the resulting

complex. But this will only be the beginning, because the actin-binding proteins form a highly interactive regulatory system. Consequently, direct tests at the cellular level will be necessary to assign physiological function. □

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Particle physics

Computer simulations of quark and gluon interactions

from D. J. Wallace

COMPUTATIONAL methods have a particularly important role to play in the many areas of science and engineering where current research and design problems are too complex to permit analytical study. The rapid expansion of international interest in numerical simulation is due in large measure to the increasing speed and memory of computers. Conversely, many problems demand far greater power than even present "supercomputers" can provide, a realization that is driving the development of yet more powerful machines. One of the most active areas is the study of the strong nuclear force, involving the numerical simulation of the interactions between quarks and gluons on a four-dimensional lattice, approximating continuous space and time.

Typically, the numerical approach adopts a discrete representation of the system under study. For example, in the case of fluid flow in systems of pipes, the velocity field $\mathbf{v}(\mathbf{r}, t)$ which depends on the spatial position $\mathbf{r}$ and time t , is approximated by the velocities $\mathbf{v}_i(t) = \mathbf{v}(\mathbf{r}_i, t)$, $i = 1, 2 \dots N$ at points $\mathbf{r}_i$ in the fluid. The differential equations obeyed by the velocity field $\mathbf{v}(\mathbf{r}, t)$ are approximated by difference equations obeyed by the $\mathbf{v}_i$. Since we now have a finite set of variables, these difference equations can be solved on a computer. Clearly, if the set of N points chosen is sufficiently dense, and if the starting equations incorporate all of the relevant physics (in this case, elastic properties, viscosity, compressibility, thermodynamic properties . . . the equations can become complicated!) then, in principle, arbitrary numerical accuracy can be obtained.

As in all cases, a key ingredient for the successful simulation of the strong nuclear force is knowledge of the fundamental equations describing the force. It is now widely accepted that these equations must be formulated not in terms of some force that binds protons and neutrons within the nucleus, but at a deeper level, in terms of the force between more fundamental constituents, out of which protons and neutrons are built. Just as the electric force binds nuclei and electrons together to form neutral atoms which can then combine to form molecules under the residual Van der Waals forces, so we now understand that the strong nuclear force binds more fundamental constituents together to form protons and neutrons; nuclei are then "molecules" formed by the residual strong force between protons and neutrons. This theory is called quantum chromodynamics (QCD) and describes how the so-called

quark constituents interact with one another through the exchange of "gluons".

Apart from experimental evidence from high energy scattering and from model calculations, QCD is aesthetically attractive because it belongs to the same class of theories as the electromagnetic and weak nuclear forces, namely it is a gauge theory. However, the usual weak-coupling perturbation theory, which has been so successfully applied to the electroweak interactions, breaks down for QCD because the effects of interactions are not small. Indeed, perturbation theory has to fail because free quarks, on which the theory would be based, are not observed experimentally; somehow they are permanently confined within observed particles such as the proton. The solution of this confinement problem in QCD should enable one to calculate the masses of the numerous "hadronic" particles such as the proton, just as properties of atoms and molecules can, in principle, be calculated in terms of the electric and magnetic forces among their nuclei and electrons.

The radical step of abandoning the continuity of space and time and formulating QCD on a discrete lattice of points was first made by K.G. Wilson of Cornell University ten years ago. In the currently most popular version of this approach, the quarks are allowed to sit at sites of a regular, usually hypercubic, four-dimensional lattice (approximating space-time) with the gluon variables occupying the links joining the adjacent sites. The theory then requires that the quark and gluon variables are allowed to interact and come to an equilibrium. One can then calculate the propagation, that is the motion on the four-dimensional lattice, of the combination of quarks that make up a particular particle. The form of the propagation depends on the mass of the particle which can hence be estimated from the numerical simulation. The whole problem is somewhat akin to, but much more complicated than, the simulation of the Brownian motion of a particle of dust in a fluid under the chaotic bombardment of the molecules in the fluid.

This is an enormously demanding computational project for many reasons, several of which are endemic in simulation problems. First, the quark and gluon variables are rather complicated; at least 50 variables must be stored at each lattice site in any realistic simulation. Second, we are interested in the limit in which the lattice spacing is very fine compared with the particle of interest. Since the computer must simulate a box of space and time large

enough to contain the proton without its feeling the edges of the box, we need to have many lattice sites. Third, just as small changes in the shape of a cloud may occur rapidly but the overall aspect tends to occur much more slowly, so it takes many steps on the computer to simulate the behaviour of a very correlated object, such as the proton, which extends over many lattice sites. Fourth, the problem is four-dimensional so that increasing the fineness of the lattice by a factor of 2 requires 2^4 more sites; in fact the problem is much worse since the proton now extends over more lattice sites, so that the correlation problem demands yet more computer time. Finally, since the quarks are fermions and must obey Fermi statistics, the quark variables cannot be directly represented by numbers and new algorithms must be developed to deal with them; to date, these are still very costly in terms of computer time.

Notwithstanding these difficulties, very considerable progress has been made in extracting strong interaction physics from simulations based on lattices, currently with a maximum of typically $16 \times 16 \times 16 \times 16$ sites. There are now good quality numerical data showing that the effective force between a quark and an anti-quark changes from the Coulomb form at separations smaller than the proton size to a constant (attractive) force at large separations; confinement then emerges because infinite energy would be required to isolate a single quark. The theoretically expected "scaling" behaviour as the lattice is made finer and finer is beginning to be exposed, to a numerical accuracy of some ten per cent. The reduction of statistical and systematic uncertainty in particle mass predictions is likely to be reduced to this level in the round of calculations now being undertaken. Practical suggestions for the calculation of particle decay and scattering have also been made.

There is a further dimension to the computational aspects that is central to the feasibility of these and many other simulations. To appreciate the point one must realize that the increase in the speed of computers by a factor of roughly a million in the past thirty years has been achieved not only because of the increasing miniaturization and speed of the electronic components but also because machines are designed increasingly to do many things simultaneously, that is in parallel. The rates of tens to hundreds of millions of multiplies per second obtained from modern computers rely on "pipelining" many multiplies through the computer, or on using many multiplying units connected in an array, so that they can communicate. The current success of most large-scale simulations is heavily dependent on the fact that the method can exploit parallel computation with close to 100 per cent efficiency; usually the reason is that the similar calculations which have to be done at every site of the lattice of points in the simulation are perfectly handled by, for

example, a communicating array of processors.

The first QCD calculations on an array machine were done by the Edinburgh group on the ICL Distributed Array Processor (DAP). The potential of arrays of microprocessors, and their relative ease of construction has already encouraged physicists to build special purpose machines for scientific calculations: for QCD, a 64-processor machine built at the California Institute of Technology is already producing interesting results and at Columbia a project is nearing fruition to build a 64-mode machine a hundred times more powerful (and probably more powerful for this work than any available commercial machine). Companies such as Floating Point Systems, Cray and CDC are

responding with projects involving configurations of several communicating supercomputers, and Denelcor also have ambitious plans. VLSI chips, such as special-purpose arithmetic processors, the GEC GRID chip, and the Inmos transputer, with self-contained processor, memory and communications, will enable massively parallel multi-processors of enormous power to be built. Since these remarks do not even touch upon IBM or Japanese intentions, there can be no doubt that the impact of parallel processing in the next few years will result in an even more rapid growth in computer power than has been previously witnessed. □

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Enzyme therapy

Treatment of inborn errors of metabolism by transplantation

from Reed E. Pyeritz

FUNCTIONAL deficiency of a specific enzyme has been found in the majority of the more than 200 inborn errors of human metabolism. Potential treatments of the resulting clinical abnormalities are nearly as numerous as the different molecular pathologies underlying these enzymopathies. The paper by P.W. Gasper *et al.* on page 467 of this issue¹ illustrates and extends one promising approach — that of bone marrow transplantation.

Some forms of treatment have been impressively effective. For example, persons with one form of cystathionine synthase deficiency become biochemically normal when treated with pharmacological doses of pyridoxine; if detected and treated early in life, they may be spared all of the severe complications of homocystinuria. Similarly, restriction of dietary protein, in particular phenylalanine, markedly ameliorates the phenotype of phenylketonuria and results in nearly normal intelligence in children otherwise doomed to profound retardation. And the invariably lethal severe combined immunodeficiency produced by a lack of adenosine deaminase can be treated effectively by partial exchange transfusions because the enzyme is present in normal erythrocytes.

The last example differs from the others as the functional enzyme is administered from an external source. This has been attempted in the treatment of numerous inborn errors, but rarely with the success achieved in adenosine deaminase deficiency. Problems with enzyme therapy are legion. They include the need for a source of adequate enzyme, for purification of non-immunogenic and non-pyrogenic enzyme at reasonable cost and for delivery of the enzyme to appropriate tissues. Moreover, even when helpful, such treat-

ment is transient at best; it is limited by the life span of the organelle or cell carrying the enzyme and by the clearance and degradation of the enzyme, or by both. Furthermore, treatment of many conditions that produce neurological deterioration or mental retardation is limited by the blood-brain barrier, which prevents active enzyme from gaining access to the central nervous system².

For these reasons, investigators have been focusing on more permanent, and ultimately more physiological, methods of enzyme replacement therapy. For some, the final goal is either the addition of a functional copy of whichever mutant gene causes the condition, or the activation of another locus in the genome that can substitute, at least in part, for the mutant one. Although many technical problems remain — including those of targeting the functional gene to the appropriate tissue and then ensuring the proper physiological regulation of its transcription — gene therapy of somatic tissue has been attempted, will undoubtedly be successful in the near future, and may become the treatment of choice for some inborn errors before the end of the decade.

Transplantation of cells, tissue or whole organs affords another method of providing a patient with a constant, and sometimes regulatable, source of enzyme. In some cases, transplantation is effective in replacing an organ damaged by the metabolic defect. For example, a normal kidney transplanted to a patient with Fabry's syndrome has enough α -galactosidase activity to protect itself from the metabolic overload of neutral glycosphingolipids it encounters, but insufficient activity to treat the entire patient³. In other disorders, transplantation of a single organ may be

sufficient to improve markedly, or even cure, the major clinical effects. Liver transplantation in a variety of conditions, such as type I glycogen storage disease⁴, Wilson's disease and α -1 antitrypsin deficiency fall into that category.

Cells of the bone marrow offer several advantages in this regard. First, the techniques of bone marrow transplantation have become standardized, predictable and reasonably effective. Second, a variety of stem cells exist in marrow, each capable of performing different, if overlapping, metabolic activities. Third, mature cells from bone marrow precursors circulate to all organs of the body. Finally, enzymes of bone marrow-derived cells should be able to function in transit to reduce the toxic metabolites carried in the plasma, and hence their deposition in peripheral tissue, although the problem of the blood-brain barrier remains. Bone marrow transplantation has been notably successful in treating a number of heritable disorders, including those that are primarily erythropoietic (sickle cell disease⁵, thalassaemia⁶), immunological (various immunodeficiency states), reticuloendothelial (Gaucher's disease⁷), a combination thereof (Wiskott-Aldrich syndrome⁸), or osteoclastic (severe osteopetrosis⁹).

Lysosomal storage disorders, in which deficiency of a single enzyme leads to ineffective catabolism of macromolecules, have long been prototypes in the study of the therapy of genetic diseases. Patients with many of the mucopolysaccharidoses and sphingolipidoses have been treated over the past decade by all of the methods noted above, except gene replacement. Some encouraging, but preliminary, success with bone marrow transplantation has been reported in lysosomal disorders that lack primary central nervous system involvement, such as Gaucher's disease⁷ and Morquio's syndrome¹⁰. However, early reports of mental improvement in Hurler's syndrome¹¹ have largely been tempered by longer experience or the development of graft-versus-host disease.

Mucopolysaccharidosis VI (MPS VI; Maroteaux-Lamy syndrome) is the result of arylsulphatase B deficiency and results in severe skeletal, cardiac and pulmonary complications while sparing intellect. Bone marrow cells produce arylsulphatase B, so bone marrow transplantation could improve the condition or, if performed early enough in life, modulate the short stature, skeletal deformity, coarse facies and infiltration of the myocardium that inevitably occurs. To investigate the potential of such therapy, Gasper *et al.*¹ took advantage of an animal model for MPS VI: cats deficient in arylsulphatase B develop clinical features similar to humans. Soon after engraftment of bone marrow from an unaffected littermate, an arylsulphatase-deficient cat showed signs of biochemical improvement. Reduced urinary excretion of mucopolysaccharide metabolites and increased leukocyte

arylsulphatase B activity persisted for the length of the study, 250 days. The most objective sign of clinical improvement was a resolution of corneal clouding, although other signs, such as the cat's ability to walk and chew, its coarse facies and demeanor, also seemed to improve. It will be of interest to determine how much clinical improvement is possible in this and similarly-treated cats, and to document the pathological improvement at the end of their substantially lengthened lives.

Animal models for inborn errors of metabolism could lead the way to a much wider use of clinical marrow transplantation. Animals will be particularly valuable for testing methods of broaching

or circumventing the blood-brain barrier in those conditions with neurological sequelae¹².

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Immunology

T-cell receptor companions

from M. J. Owen

WITH the molecular analysis of the T-cell antigen receptor, molecular biologists have recently begun to solve the outstanding immunological question of how T cells recognize antigens and thus contribute towards the antigen-specific immune response. As already described in these columns¹, the receptor is composed of a β chain, which was well defined some months ago, and an α chain whose partial amino acid sequence^{2,3} has recently confirmed a tentative identification from cDNA clones^{4,5}. But the heterodimer formed by these two chains can, so far as is known, subserve only the recognition function of the molecule. Some other structure, therefore, must be responsible for transducing the signal to the interior of the cell and initiating the cellular response to antigen. An obvious candidate for this function is the so-called T3 molecule, which is physically associated with the receptor heterodimer⁶. T3 is a glycoprotein complex of three polypeptide chains (γ , δ and ξ). It, too, is beginning to yield to the all-embracing techniques of molecular biology as shown, in particular, by a paper from Terhorst's laboratory on page 413 of this issue⁷.

The T3 glycoprotein is unlikely to play a direct role in specific binding of antigen but is nonetheless probably obligatory for receptor function and may play a part in the stabilization of the initial interaction between receptor and antigens, as well as in signal transduction⁸. In this regard it is intriguing that calcium fluxes can be induced in T cells by antibodies against the T3 glycoprotein⁹. Furthermore, the expression of T3 is apparently linked to that of the antigen receptor, since in no case has one been detected at the T-cell surface in the absence of the other.

To clone the δ chain of T3, Terhorst's group has used the protein sequence route in which oligonucleotide primers are synthesized on the basis of a partial protein

sequence and used as probes with which to extract the desired cDNA species. (It is worth pointing out for the aficionados that the hybridisation probe used was a 19-mer and 15-mer mixture, consisting of 576 and 128 different sequences, respectively; this must represent a record for the redundancy of a primer used successfully in a cDNA cloning exercise.) The deduced primary sequence of the T3 δ chain confirms much of the biochemical data reported by Terhorst's group and by others, and contains few revelations or surprises. Predictably, it shows no sequence homology with members of the multi-gene family that includes the genes for the T-cell receptor, immunoglobulins and the major histocompatibility antigens. One point of interest is that the human δ -chain probe cross-hybridizes with a mRNA species in mouse T cells (as detected by Northern blotting analysis), which represents the first convincing demonstration of a murine equivalent of at least part of the T3 complex. The failure to identify a T3-like molecule on the surface of mouse T cells by means of serology has been a point of some concern to those who advocated a pivotal role for T3 in the T-cell response to antigen.

One possible clue to the mechanism of association of the α - β dimer with the T3 complex has emerged from this study. Terhorst's group point out that the transmembrane segments of the α and β chains of the structure both contain a lysine residue whereas an aspartic acid side chain is located in the corresponding region of the δ -chain transbilayer peptide. The ion pair that would form between these residues in the hydrophobic environment of the lipid bilayer would provide a strong stabilizing force for the T3-receptor complex. If this speculation is correct, *in vitro* mutagenesis experiments in which the transmembrane charged residues are altered should prove rewarding.

The minimal array of proteins involved

in the response to antigen on the surface of an effector T cell seems to be the T3-receptor complex, the T4 or T8 glycoproteins, and possibly the mysterious protein described by Tonegawa's group and originally thought to be an α chain candidate¹⁰. The availability of clones encoding all of these surface structures will be necessary before molecular immunologists can begin to manipulate the system and provide a complete molecular description of the recognition and activation processes. The isolation of a cDNA clone encoding the δ chain is an essential first step towards defining the role of T3. The partial protein sequence of the T3 ξ chain reported on page 455 of this issue¹¹ should provide the basis for the cloning of its cDNA. And the deduced protein sequence of this chain will prove especially interesting since it is postulated by Terhorst and his colleagues to be a constituent of a putative calcium channel involved in the process of activation of the T cell. At the current rate of progress, it should not be long before the sequence is reported and can be commented on in these columns.

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100 years ago

OVER-PRESSURE IN ELEMENTARY SCHOOLS

Take the typical case of a class of seventy children, starting with about the same attainments. The bulk of these will be average boys, or girls, as the case may be, fairly healthy and intelligent, not given to over-much study, but still ready to fall in with the requirements of the school. But there will also be some half-starved children who often come without any breakfast, dull children — descendants of a wholly uncultured race — feeble children, and others averse to any restraint and constitutionally irritable, together with childer who are weary with toil at home. Besides these there are the exceptionally clever children, who are in danger of under-pressure and the over sensitive or ambitious who are prone to over work themselves. It is evident that the general scope of the instruction must be adapted to the average of the class.

From *Nature* **31**, 73; 27 November 1884.

Haematopoietic growth factors

SIR — May we add some important points to those made by T.M. Dexter in his News and Views article on haematopoietic growth factors¹. The development of blood cells requires a programme for the multiplication of stem cells and their differentiation to various types of mature cells with different functions. The mature cells no longer multiply. The normal developmental programme also has to include a mechanism for coupling the induction of multiplication in stem cells to the induction of differentiation, so as to maintain the normal balance between undifferentiated and mature cells. The points we wish to add are that multiplication (growth) and differentiation of blood cells are induced by different proteins, those that induce growth and those that induce differentiation, and that coupling between growth and differentiation can be achieved by a growth factor switching on production of a differentiation factor.

In cells of the myeloid series there are four different growth-inducing proteins. These are now called colony stimulating factors (CSF), or macrophage and granulocyte inducers — types 1 (MGI-1)^{1,4}. Of the four growth factors, one (M7) induces the development of clones with macrophages, another (G7) clones with granulocytes, the third (GM) clones with both macrophages and granulocytes, and the fourth (also called interleukin-3), clones with macrophages, granulocytes, eosinophils, mast cells, erythroid cells, or megakaryocytes. This multigene family represents a hierarchy of growth factors for different stages of blood cell development as the precursor cells become more restricted in their developmental programme. How do normal myeloid precursor cells induced to multiply by these growth factors develop into clones that contain mature differentiated cells that stop multiplying when they terminally differentiate? This has been answered by experiments that complement those discussed by Dexter¹.

Normal myeloid precursors require addition of one of the myeloid growth factors for cell viability and multiplication. But there are myeloid leukaemic cells that are viable and multiply without adding growth factor, and that can be induced to differentiate to non-malignant terminally differentiated macrophages or granulocytes by proteins which are different from the growth factors called CSF or MGI-1^{2,3}. These differentiation-inducing proteins, which also induce differentiation of normal myeloid cells^{2,3}, have been called MGI-2^{2,5} or differentiation factors (DF)^{6,7}. The injection of these MGI-2 proteins into leukaemic mice inhibits the development of myeloid leukaemia^{3,8}. Normal myeloid precursor cells cultured with one of the myeloid growth factors endogenously produce the differentiation factor MGI-2^{2,3,9} and this serves as an efficient mechanism to

couple growth and differentiation. Differences in the time of the switch-on of the differentiation factor can produce differences in the amount of multiplication before differentiation. There is more than one type of MGI-2^{5,10}. Different growth factors may switch on different differentiation factors and this may determine the differentiated cell type.

It has been suggested from experiments with WEHI-3B myeloid leukaemia cells that one of the myeloid growth factors (MGI-1G = G-CSF) can also act as a differentiation factor for these leukaemic cells⁴. This has not so far been found with other clones of myeloid leukaemic cells, and studies with these other clones have identified MGI-2 proteins that have no MGI-1G activity^{3,5-7}. In addition, MGI-2 but not MGI-1 binds to double stranded DNA¹¹. The form of MGI-1G used in the experiments with WEHI-3B may be a hybrid molecule, but there is also a different explanation. WEHI-3B cells constitutively produce their own growth factor and under appropriate conditions can be induced to endogenously produce their own differentiation factor¹¹. As with normal myeloid precursors, WEHI-3B cells may thus be induced to differentiate indirectly by the growth factor MGI-1G (G-CSF). In addition to these results with myeloid cells, different proteins that induce growth and differentiation have been found with B lymphocytes¹² and this presumably also applies to the other types of blood cells. Induction of differentiation factor may also play a role in the normal developmental programme of other cell types.

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Calibrations for nuclear winter

SIR — In their recent complaint¹ about John Maddox's criticisms^{2,3} of their climate modelling work on the 'nuclear winter' concept⁴, Turco *et al.* attempt to buttress their case by stating that the climate model they used was partly calibrated by (1) 12 years of research on

martian dust storms, (2) the climatic consequences of volcanic explosions on Earth, and (3) the possible collision of an asteroid or cometary nucleus with Earth at the time of the Cretaceous/Tertiary extinctions. In addition, they say that their work was reviewed by a large number of experts and that it referred to many previous studies. If these reasons are indeed the basis for their confidence, the criticism they received was well justified.

To begin with, what good is a technique that is only *partly* calibrated? And how can a model be calibrated against a *possible* phenomenon which may or may not have actually occurred, and at that in the distant past? With respect to the climatic consequences of volcanic explosions, Turco *et al.* indicate in their complaint that these are caused primarily by sulphuric acid aerosols and not by the smoke and dust that is supposed to operate in the nuclear winter scenario. So how can this comparison be of any use? In addition, of what real comparative value is the planet Mars? It has no liquid water on its surface, while Earth is 70% covered by seas; and its atmospheric mass is miniscule. Dust there operates almost as if it were in a vacuum; and, again Turco *et al.* claim that it is not dust but rather sooty smoke from fires that is the major cause of nuclear winter. And as for citing lots of background material and getting the opinions of a large number of experts, what does that *prove*? Absolutely nothing, which is precisely the point made by John Maddox, and one which I heartily endorse.

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DNA methylation and G + C rich DNA

SIR — In a recent 'News and Views' article, Max collated evidence showing that the 5' region of certain genes is undermethylated in the cells of all tissues examined including sperm¹. This was used to explain the recent observations² that CG dinucleotides are present in unusually high amounts in certain regions of some genes. Where cytosine methylation does not occur, deamination of a cytosine base in DNA will produce uracil which can be removed from the DNA and the original sequence re-established. However, the occasional deamination of methylcytosine to thymine in DNA will lead, over many generations, to the loss of CG dinucleotides from the genome — so called CG suppression³. McClelland and Ivarie⁴ found a reduced suppression in the 5' flanking regions of some vertebrate genes which might imply that these regions are

undermethylated in the germ line. More recently Erickson⁵ has pointed out that several genes are highly methylated in sperm DNA, a finding which does not support the idea that in these cases a lack of methylation is the cause of the reduced CG suppression.

There are two ways to explain a lack of CG suppression in a particular region. As suggested by Max and explained above this would result if the CGs in that region are not methylated. It would also result if methyl CGs are not deaminated in that region and we have recently presented evidence to support this mechanism⁶. An analysis of various regions of vertebrate genomes demonstrates that CG dinucleotides occur at the expected frequency wherever G + C rich regions occur. (G + C rich regions having a composition of greater than 60% G + C.) The increased stability of the double helix in G + C rich regions will mitigate against the deamination reaction which requires the DNA to be single stranded. Even if heavily methylated, we would predict that CG dinucleotides will be maintained in these very G + C rich regions of DNA and that is what is found.

Deamination of methylcytosine in CG dinucleotides will lead to a decrease in the G + C content of DNA³ but where these dinucleotides are maintained no change in base composition would be expected. A + T rich regions should remain A + T rich and there is no relevant reason why they should become G + C rich. This implies that those regions which fail to show a suppression of CG dinucleotides have been rich in G + C for a very long time and this may be why they are resistant to deamination. Other events may lead G + C rich regions to mutate to a more average composition which may then allow methylcytosine deamination to occur, thereby accelerating the change to an A + T rich composition. Where a high G + C content is maintained or arises either by chance or because of selection it shows little CG suppression.

CG dinucleotides are highly suppressed in regions of the vertebrate genome containing less than 40% G + C and such regions have little remaining scope to exhibit further base changes as a result of methylcytosine deamination. Such mutations are expected to occur only in regions of intermediate composition where a number of methyl CG dinucleotides still remain in an environment conducive to deamination.

We do not argue against the proposal that lack of cytosine methylation may be the cause of the lack of CG suppression in some cases and indeed Cooper *et al.*⁷ have shown that short stretches of unmethylated DNA are present in the vertebrate genome. Nonetheless, we are of the opinion that a simple failure to deaminate methylcytosine in G + C rich regions may be a more general explanation for the absence of CG suppression in G + C rich regions of the vertebrate genome.

We thank Dr Max for his comments on this letter and Dr Bird for helpful discussions.

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Germ-line gene therapy a misnomer?

SIR — I would like to add to the medical and deontological reservations raised by M.E. Pembrey¹ regarding gene therapy. Two modes of gene therapy are considered: one involving intervention at the level of somatic cells on their immediate precursors (such as bone marrow cells²) and the second, modification of the germ line level³. The commonly implied advantage of germ-line gene therapy is the correction of the defect could be made transmissible to further generations.

But, at present, there is no way to control or target the site of gene insertions, which seem to occur at random⁴. Consequently, deficient and inserted loci will, in most cases, lie on different chromosomes (or be separated by a large distance on the same chromosome) and so will segregate more or less independently. Thus, given a homozygous recessive defect, and as long as only a single chromosome can be the target for insertion, descendants will have only a 50% chance of inheriting the correcting locus along with the defective locus. That is, the so-called germ-line gene therapy will effectively control a genetic disease in the corrected individual, but the defective locus and potential for disease will still be transmitted to the progeny. Evidence is also accumulating demonstrating that this randomness of insertion bears a significant mutagenic potential⁵. Multiple independent insertions of the correcting locus would thus not be a solution to this problem, since although increasing the likelihood that offspring receive a "correcting" locus, they would also increase the likelihood of insertional mutagenesis.

Thus, at present, germ-line gene therapy of an individual would not eliminate the necessity to reiterate this therapy in his offspring, since diagnosis for the absence of the correcting gene in the offspring would have to be delayed to the embryo stage with present techniques — too late to apply germ-line gene therapy *de novo*. Arguments for germ-line intervention should thus take into consideration that the benefits are mostly restricted to one gener-

ation only. The possibility of insertional mutagenesis, in fact, seems to suggest that germ-line therapy is more likely to increase future genetic problems than it is to cure them.

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Three-dimensional chemical waves in metals

SIR — The review article by Winfree and Strogatz¹ mentions rotating spiral waves in a modified Belousov² chemical reagent, in a monolayer of *Dictyostelium discoideum* cells responding to a pulse of cyclic adenosine monophosphate³ and in successive photographs of the retina of a chicken⁴.

The photograph of a scroll wave in modified⁵ Belousov-Zhabotinsky reagent is reminiscent of the mechanism by which dislocations may multiply in metals first suggested by Frank and Read⁶. In this model, a line representing a dislocation in a three-dimensional dislocation network in a crystal is successively deformed by a shear stress so as to produce ring dislocations. An unlimited amount of slip may be produced.

The stress required for this process is of the order of Gb/l where G is the shear modulus, b is the Burgers vector, and l is the length of the line. Typically, for a metal $G = 500$ GPa, $b = 0.25$ nm and $l = 100$ μ m. This gives a stress of the order of 10^{-6} pascals and an energy of the order of 10^{-10} joules per metre of dislocation line. This corresponds to an energy of approximately 10 eV per atom along the dislocation line.

The value of 10 eV is in good agreement with the energy of a typical chemical bond and thus the initial dislocation line may be seen as an organizing centre for the generation of ring dislocations which are analogous to chemical waves travelling through the medium.

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Multiple specific contacts between a mammalian transcription factor and its cognate promoters

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The human transcription factor Sp1 binds upstream of certain viral and cellular promoters and activates initiation of RNA synthesis from these promoters by RNA polymerase II. The Sp1 binding sites of both simian virus 40 and a related monkey promoter contain multiple copies of the sequence GGGCGG, three or four of these sequences forming close contacts with Sp1. The clustered contacts fall on one strand of the DNA and are arranged similarly in the major groove of the DNA helix in both promoters.

MOST promoters for eukaryotic protein-coding genes contain distinctive upstream control sequences required for gene expression. Although the molecular mechanism by which these upstream sequences act to modulate transcription is not understood, it probably involves promoter-specific protein factors that interact with the DNA and influence the core transcription machinery to initiate RNA synthesis from particular sites.

Recently, two such promoter-specific transcription factors have been isolated and characterized: one, Sp1, interacting specifically with upstream control sequences of the simian virus 40 (SV40) promoter^{1,2} and a second, HSTF, involved in transcription of *Drosophila* heat shock genes³. In both cases the factors have been partially purified from cell or tissue extracts, their respective bind promoters and activate transcription *in vitro*. The stimulation of SV40 transcription by the Sp1 factor is dependent on sequences within a region of 21-base pair (bp) direct repeats located 48–103 bp upstream from the start sites for early transcription (ref. 2; D.G. and R.T., unpublished data). Sp1 binding activates transcription of both viral strands; sequences within the binding region are required for *in vivo* and *in vitro* transcription^{4–10}.

Because Sp1 is found in uninfected cells, it seems likely that it plays a role in the expression of cellular as well as viral genes. We have discovered recently a strong binding interaction between Sp1 and two binding sites occurring in a 450-bp region of the monkey genome (W.S.D. *et al.*, unpublished data). This putative monkey promoter sequence is homologous to sequences in the SV40 promoter region^{11,12}; it also directs transcription *in vivo* both in its normal chromosomal location and in an expression vector¹³.

A comparison of Sp1 binding regions in SV40 with those from the host cell should facilitate identification of specific features involved in binding and transcription. The boundaries of the SV40 and the two monkey Sp1 binding regions have been identified by DNase footprinting (ref. 2; W.S.D. *et al.*, unpublished data). The overall lengths of the regions range from 18 to 60 bp and each is associated with direct repeats of 14–21 bp. The prominence of the sequence 'GGGCGG' (the GC box) repeated many times in all three binding sites suggests that it has an important role in the recognition of the promoter region by Sp1. The ability of the footprinting technique to resolve specific features of the binding interaction is rather limited, however, in particular precluding identification of the nucleotide residues within the protected region likely to make specific contacts with the protein.

In the present experiments, we have used dimethyl sulphate to probe the binding of Sp1 to specific sites in both the SV40 and monkey genomic sequences. Dimethyl sulphate is a reactive electrophile methylating the N-3 positions of adenine and the

N-7 positions of guanine in duplex DNA¹⁴. Because dimethyl sulphate is a small molecule, protection from methylation can be used to infer the location of specific A or G residues forming intimate contacts with sequence-specific binding proteins¹⁵. By observing the pattern of dimethyl sulphate protection in the presence of Sp1, we have identified the particular GC boxes that appear to make close contact with this factor and have constructed a model for the internal structure of the binding sites.

Sp1 binding to SV40 promoter

We have shown previously that a fraction containing Sp1 from cultured human cell extracts is capable of protecting a site in the SV40 promoter region from DNase attack². In those experiments, a duplex DNA fragment was labelled at one end, incubated with Sp1 and subjected to limited cleavage with DNase I. The presence of specifically-bound Sp1 results in a gap, or footprint, in the ladder of cleavage products revealed by electrophoresis. The footprint is seen on both strands and is suppressed in the presence of excess unlabelled SV40 DNA². Partial or full protection extends ~60 bp (nucleotides 45–104), overlapping the 21-bp repeated elements and containing GC boxes II–VI and a portion of I (Fig. 1a).

To obtain a more detailed picture of the binding interaction between Sp1 and the SV40 sequences, we performed dimethyl sulphate methylation protection experiments (Fig. 1b). Sp1–DNA complexes were allowed to form at 0°C, then subjected to partial methylation with dimethyl sulphate as described in Fig. 1 legend. Methylated guanine residues were identified by cleavage of the DNA backbone¹⁵. In the presence of Sp1, 13 guanine residues on the late RNA sense strand (see ref. 16) of the SV40 control region showed specific and almost complete protection from methylation by dimethyl sulphate. These residues fall into three clusters corresponding to GC boxes III, V and VI. An additional four residues contained within the second GC box proximal to the sites of early transcription (GC box II) were weakly protected from methylation by Sp1 binding and there was also very slight protection in GC boxes I and IV. In addition, several guanine residues located near the clusters of protected residues were hypermethylated, perhaps because the local environment of bound Sp1 favours the methylation reaction.

In order to identify adenine as well as guanine residues that may be protected from methylation, Sp1 was incubated with DNA and treated with dimethyl sulphate as before, but the conditions of subsequent treatment were such that DNA was cleaved at both the N-7 methyl guanine and the N-3 methyl adenine¹⁵. In contrast to the pattern of protection seen with late-strand guanine residues, there was no significant methyla-

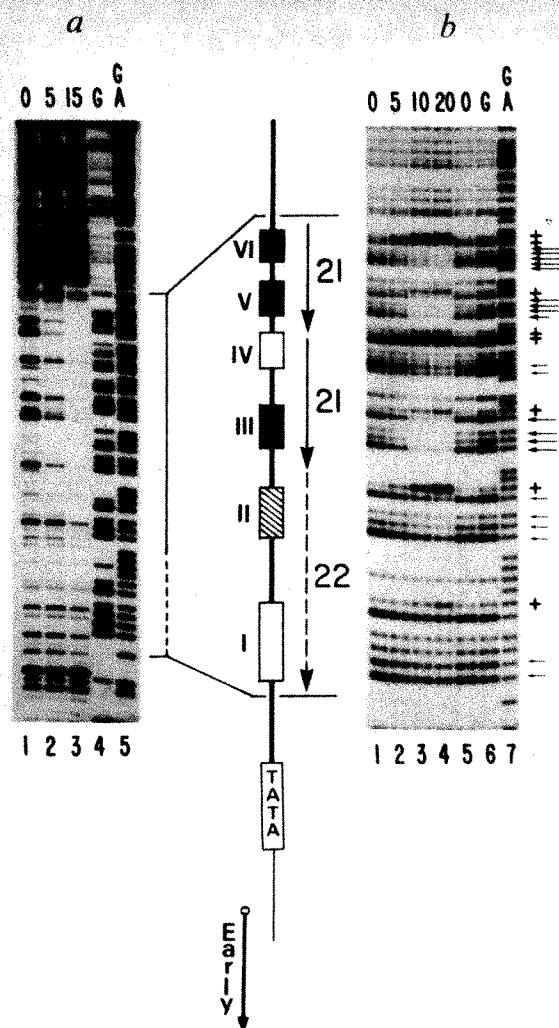


Fig. 1 Sp1 binding to the SV40 promoter region. *a*, DNase I footprinting. Reactions contained 0, 5 or 15 μ l of Sp1 as indicated. Brackets mark the limits of the protected region. Lanes G and G+A contain Maxam-Gilbert²⁰ sequence ladders prepared from the same fragment used for the binding. *b*, Dimethyl sulphate methylation protection. Reactions contained 0, 5, 10 or 20 μ l of Sp1. Arrows, protected residues; +, hypermethylated guanine residues. The central diagram schematically represents the Sp1 binding region. Rectangles, the six GGGCGG motifs in the SV40 21-bp repeat region. Filled, hatched and open rectangles represent strong, partial and weak protection from dimethyl sulphate, respectively. The 'TATA' box and direction of early transcription are also indicated.

Methods: Sp1 was partially purified by fractionation of a human (HeLa) whole-cell extract as described previously¹. The end-labelled DNA probe for binding experiments was prepared from the plasmid pX8, containing the SV40 genome with a *Xho*I linker inserted at the *Bgl*I site in the origin of viral replication⁵. The plasmid was digested with *Xho*I, labelled with [γ -³²P]ATP and polynucleotide kinase and digested again with *Pvu*II. The fragment containing the Sp1 binding site was gel-purified; this fragment is singly 5' end-labelled at a site downstream from the early 'TATA' sequence. In each binding reaction, 3 ng (17 fmol) of labelled probe fragment and 1,000 ng of unlabelled sonicated calf thymus DNA were incubated with 0–20 μ l Sp1 (0–10 ng total protein) for 15 min at 0°C in 50 μ l of the reaction buffer described by Dynan and Tjian². For DNase footprinting²¹ the reaction was warmed rapidly to room temperature and the DNA cleaved as described previously². For dimethyl sulphate methylation protection¹⁵, 1–4 μ l dimethyl sulphate was added and incubation continued for 1 min at 0°C. The reaction was terminated and DNA subjected to cleavage at sites of N-7 guanine methylation (described by Maxam and Gilbert²⁰). All samples were analysed on a denaturing 8% polyacrylamide sequencing gel.

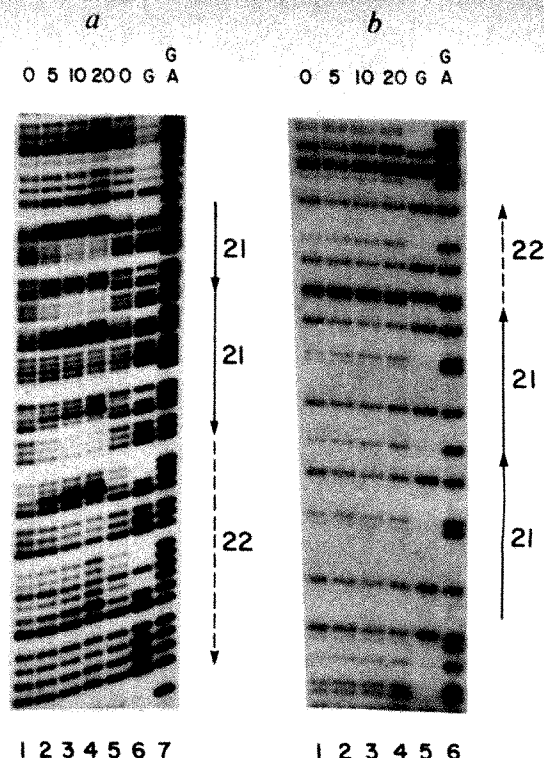


Fig. 2 Comparison of the dimethyl sulphate methylation protection pattern on opposite strands of DNA in the SV40 early promoter region. *a*, DNA probe 5'-end-labelled on the *Xho*I site downstream from the early 'TATA' box, prepared as described in Fig. 1 legend. *b*, DNA probe 5'-end-labelled at an *Eco*RI site upstream from the early promoter, prepared from the plasmid pSV07 as described by Dynan and Tjian². In both experiments, end-labelled DNA probe and unlabelled carrier DNA were incubated with 0–20 μ l Sp1 and treated with dimethyl sulphate as described in Fig. 1 legend. Simultaneous cleavage of N-7 methylated guanine and N-3 methylated adenine residues was carried out according to Gilbert *et al.*¹⁵. The Maxam-Gilbert G and G+A sequence ladders are shown alongside the dimethyl-sulphate protection pattern. Arrows, positions of the 21-bp repeats and the 22-bp sequences of the SV40 promoter.

tion protection or enhancement observed with adenine residues (Fig. 2a). No protection of either guanine or adenine was seen with an early-strand probe (Fig. 2b). Thus, Sp1 methylation protection appears to be limited to moieties in the major groove (N-7 of guanine but not N-3 of adenine) and to one strand of the DNA.

Sp1 binding to monkey promoter

The presence of Sp1 in uninfected cells suggests that a class of SV40-like cellular promoters may also be under the control of this transcription factor. We have recently characterized transcription and Sp1 binding properties of a cloned genomic monkey DNA sequence that exhibits *in vivo* and *in vitro* promoter activity (ref. 13 and W.S.D. *et al.*, unpublished data). DNase footprint analysis of the monkey DNA revealed two distinct and separate binding regions for Sp1, designated α and β (see map in Fig. 3). Sequence analysis of these two regions indicates that both contain multiple GC boxes arranged similarly to the SV40 21-bp repeat region.

We chose to focus on the β region for dimethyl sulphate methylation protection experiments both because it is located between divergent *in vivo* transcriptional start sites and because preliminary experiments showed a more distinct pattern of protection than in the α region. Side-by-side DNase footprinting and dimethyl sulphate methylation protection experiments are shown in Fig. 3a and b. The pattern of protected guanine residues in the monkey DNA is very similar to the SV40 promoter. Three of the six GC boxes on one strand of the duplex binding site were almost completely protected by Sp1 from

Fig. 3 Sp1 binding to the putative monkey promoter. **a**, DNase footprinting. Probe DNA was 5' end-labelled at a linker adjacent to an SphI site located to the right of the β binding region. Brackets, region protected by Sp1. **b**, Dimethyl sulphate methylation protection using the same probe as **a**. DNA was cleaved at methylated guanine residues. Arrows, protected guanine residues; +, hypermethylated guanine residues. A schematic representation of the protected guanine clusters within the Sp1- β binding region is shown alongside **a** and **b**. The six GC boxes are represented by solid or open rectangles, indicating residues strongly protected from dimethyl sulphate or not protected, respectively. **c**, Dimethyl sulphate methylation protection of the opposite strand. Probe DNA was 5' end-labelled at the AvaI site to the left of the Sp1 binding regions. DNA was cleaved simultaneously at methylated guanine and adenine residues. **a-c**, Binding reactions and cleavage procedures as described in Fig. 1 legend. **d**, The two distinct binding regions for Sp1, α and β , detected by DNase footprinting (W.S.D. *et al.*, unpublished data). Filled rectangles, the GGGCGG sequence motifs in each binding region. Arrows, the 17-bp repeats and the E ('early') and L ('late') transcription directions. DNA probes in these binding experiments were prepared from the plasmid p7.02, provided by Drs J. Saffer and M. Singer.

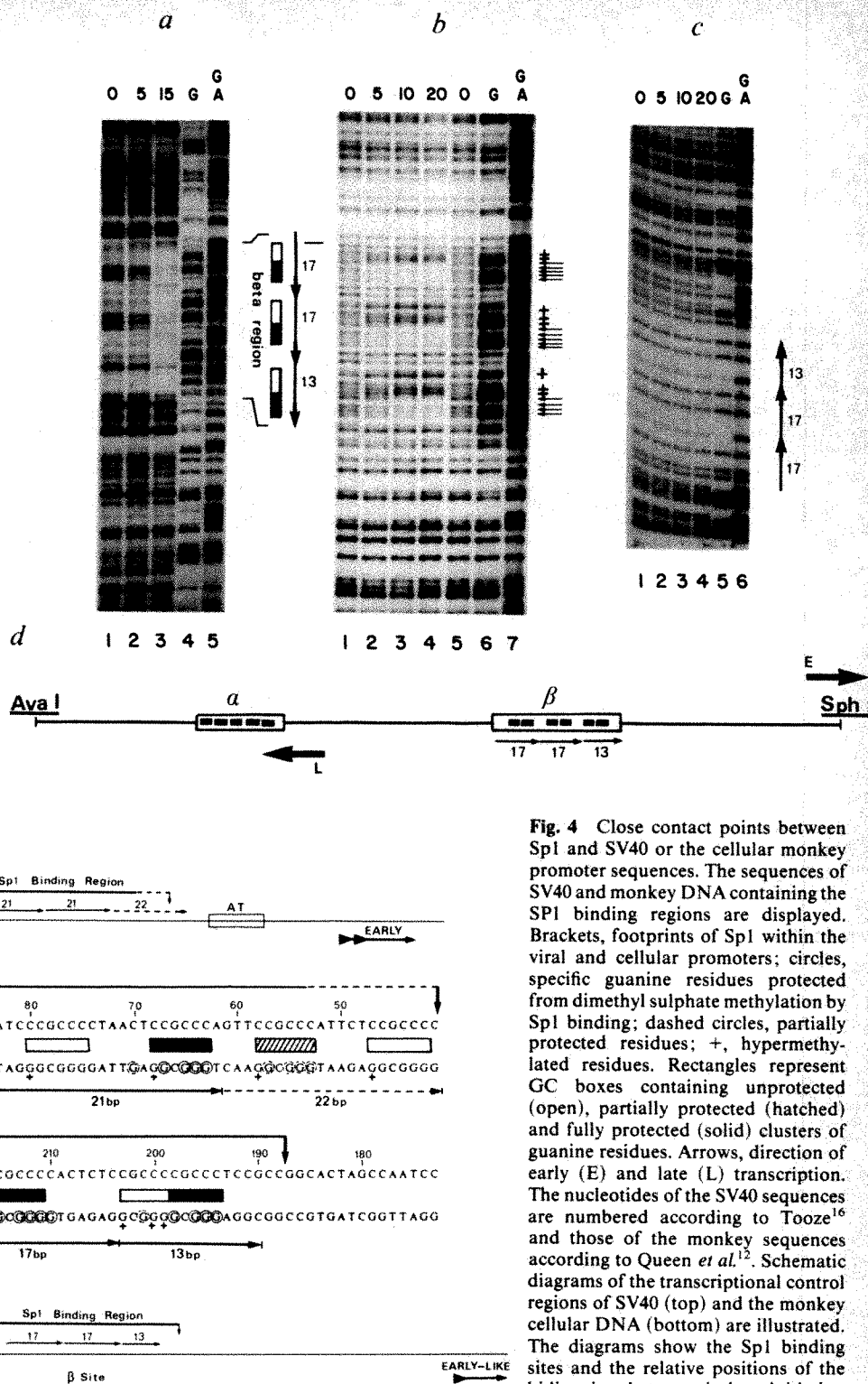


Fig. 4 Close contact points between Sp1 and SV40 or the cellular monkey promoter sequences. The sequences of SV40 and monkey DNA containing the Sp1 binding regions are displayed. Brackets, footprints of Sp1 within the viral and cellular promoters; circles, specific guanine residues protected from dimethyl sulphate methylation by Sp1 binding; dashed circles, partially protected residues; +, hypermethylated residues. Rectangles represent GC boxes containing unprotected (open), partially protected (hatched) and fully protected (solid) clusters of guanine residues. Arrows, direction of early (E) and late (L) transcription. The nucleotides of the SV40 sequences are numbered according to Tooze¹⁶ and those of the monkey sequences according to Queen *et al.*¹². Schematic diagrams of the transcriptional control regions of SV40 (top) and the monkey cellular DNA (bottom) are illustrated. The diagrams show the Sp1 binding sites and the relative positions of the bidirectional transcription initiation sites.

dimethyl sulphate attack (Fig. 3b) and hypermethylated guanine residues were found at positions directly adjacent to the methylation protection sites. No adenine residues were hypermethylated or protected on this strand (data not shown), nor was there any perturbation of methylation at guanine or adenine on the opposite strand (Fig. 3c).

Sp1 contacts on DNA helix

Data from the viral and cellular binding regions together suggest that Sp1 forms close contacts with only a selected group of guanine residues within the GC boxes. The protected atoms lie within the DNase I footprints and are all on one strand of the

duplex DNA (Fig. 4). No adenine residues are protected from modification, indicating that the contact points are located in the major groove of the double helical binding region. Although the precise nucleotide sequence and the spacing of the GC boxes are not identical in the viral and cellular promoter regions, the general pattern of close contact residues follows a similar spatial arrangement.

We have visualized the arrangement of Sp1-DNA close contact points in computer-generated stereoscopic images (Fig. 5). Each cluster of protected atoms occupies one half-turn of the DNA helix. Although protein structure cannot be predicted from methylation protection data alone, this elongated pattern of

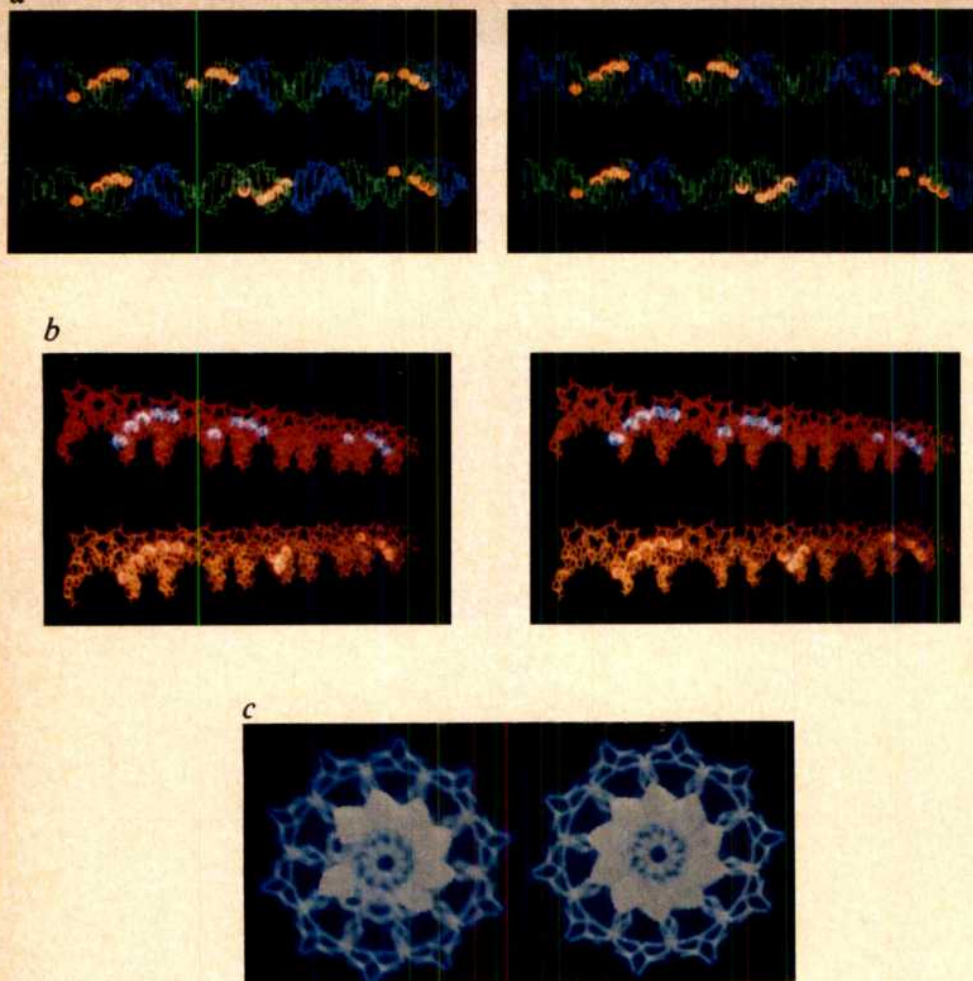


Fig. 5 Stereoscopic representations of Sp1 binding regions within SV40 and the cellular monkey promoter sequences. The two Sp1 binding regions have been aligned for maximum homology. *a*, Side view of SV40 (top helix) and monkey (bottom helix) binding sequences. The DNA sequences projected correspond to the approximate limits of the DNase footprint region. The GGGCGG sequences are highlighted in green and the surrounding DNA sequences represented in blue. The yellow spheres designate the van der Waals' radii of the guanine N-7 residues in close contact with Sp1 and strongly protected from methylation. *b*, SV40 (red helix) and monkey (orange helix) Sp1 binding regions viewed at an oblique angle to accentuate the location of close contact points within the major groove of the DNA helix. *c*, SV40 (left) and monkey (right) binding sequences are viewed down the axis of the helix. The positions of contact points with Sp1 are shown as white spheres around the DNA axis. The Sp1-DNA contacts were projected in three dimensions with the assistance of Dr Judith McClarin at the Computer Graphics Laboratory (University of California, San Francisco) as described by Jones and Tjian²².

protection is consistent with that expected if a rigid rod were laid in the major groove parallel to the DNA strands. This model is similar to that proposed for certain prokaryotic sequence-specific DNA-binding proteins, such as phage repressors, where an α -helical protein domain is believed to lie across the DNA in the major groove (see ref. 17 for review). Sp1 differs from these other proteins, however, in that only one strand of the DNA shows evidence of protein contact.

There is an interesting difference in the internal geometry of the Sp1 binding regions in SV40 and monkey DNA. In SV40, the protected residues in all three clusters are located predominantly on one face of the helix (Fig. 5*a*, top). By contrast, because of a 6-bp difference in the spacing, the centre contact cluster in the monkey β binding region is rotated relative to SV40, such that these residues are now on the bottom face of the helix. This difference in geometry can be seen readily in Fig. 5*b* and *c*. Interestingly, because of redundancy in the monkey sequence, alternate contacts are available at the top of the helix in a position fully homologous to those in SV40, but apparently are not used. This preference could arise from steric considerations as bulky binding moieties may not be accommodated easily in the restricted space between protected clusters. As a consequence of these possible steric interactions, the DNA helix may be strained in SV40 but not in monkey DNA. There is some experimental evidence to support this view. Our previous studies have shown the presence of several specific sites of enhanced DNase I cleavage in sequences just outside the footprint region in SV40, whereas such sites are not found in sequences flanking the monkey β Sp1 binding region (ref. 2; W.S.D. *et al.*, unpublished data).

Our results confirm the notion that structural moieties within the repeated GC boxes make a significant contribution to the sequence-specific binding of Sp1. However, one of the more striking findings from the dimethyl sulphate protection experi-

ments is that the GC boxes are markedly nonequivalent. This implies either that sequences outside the boxes are important in determining the course of binding, or that the placement of the boxes with respect to each other favours certain interactions at the expense of others. It is interesting to note that GC box IV, which lies in the middle of the SV40 binding region but is not protected from dimethyl sulphate, has been shown previously to contain a site hypersensitive to DNase I cleavage².

Strand specificity and multiple binding sites

The fact that we have observed binding only to one DNA strand may reflect bias inherent in our technique, because the reactive moiety we have examined in the major groove is found only in guanine and because the late-sense strand of the DNA is significantly more guanine-rich than the early-sense strand. However, the one guanine residue in the early strand of each GC box was never protected from methylation. This degree of strand specificity has not been observed with most prokaryotic DNA binding proteins, although there is some precedent among other eukaryotic proteins. Transcription factor IIIA, for example, which binds to and activates the genes for 5S RNA, makes contact primarily with the nontemplate strand of the DNA¹⁸.

Previous observations have shown that Sp1 binds to an extended and internally repetitious region in both the viral and cellular DNAs. The overall length of the DNase footprint can be as little as 18 bp of complete protection in the monkey α site and as much as 55 bp in the monkey β site (ref. 2; W.S.O. *et al.*, unpublished data). In addition to this length heterogeneity, the dimethyl sulphate protection experiments indicate that the internal structure of the binding region varies also. In particular, the rotation of the central contacts in the β site relative to SV40 suggests a degree of flexibility in the final conformation of the Sp1-DNA complex.

These findings support the notion that some binding regions may accommodate multiple protomers of Sp1, with each protomer interacting independently with a subset of sequences in the region. The existence of such individual sites can be tested using mutants altered in single GC boxes or variants naturally containing only one or two GC boxes. We have begun to analyse a set of such DNA templates, some of which were generated by oligonucleotide site-directed mutagenesis of the Sp1 binding region in SV40. Preliminary results support the multi-site model (D.G., H. Barerra-Saldana, K. Takahashi, P. Chambon and R.T., unpublished results; K. A. Jones and R.T., unpublished results; W.S.D. *et al.*, unpublished results).

Sp1 binding and transcriptional activation

Unlike the recognition sites for prokaryotic transcription factors such as phage repressors or the cyclic AMP receptor proteins, the Sp1 binding regions contain no sequence palindromes and are markedly asymmetric in base composition. Despite this asymmetry, there is good evidence that the Sp1-dependent transcription of SV40 occurs on both strands of the DNA. Addition of Sp1 to an *in vitro* transcription reaction results in the appearance of two transcripts that initiate on opposite strands at locations approximately equidistant from the centre of the binding region¹. Inversion of the SV40 21-bp repeats results in a promoter remaining competent to direct viral RNA synthesis in the early direction *in vivo*⁷.

We are thus left with a paradox in which binding of a factor

to an asymmetric sequence results in symmetric activation of transcription. If activation proceeds by protein-protein contact, similar protein recognition sites would then occur at either end of an asymmetric molecule. If, however, the Sp1 effect proceeds via a perturbation of the helix, one might expect not only that the effect on transcription could propagate in both directions, but also that some variation could be tolerated in the distance between the binding site and the start of transcription. In support of this idea, alteration of sequences in the 21-bp repeats has been reported to affect late transcription from sites as far away as 200 bp^{5,9,10}.

There is no direct evidence that Sp1 is bound to DNA *in vivo*. In one recent DNase hypersensitivity study, however, SV40 minichromosomes appeared to have a small protected region in the 21-bp repeats, flanked by regions of hypersensitivity extending to either side¹⁹. Final proof of the *in vivo* role of Sp1, as well as a more complete understanding of the nature of the binding interaction, awaits identification of the active molecule and development of improved techniques for measuring its interaction with the chromatin template *in situ*.

We thank J. Saffer and M. Singer for the gift of cloned monkey DNA and for communicating unpublished results; J. McClarin for assistance with computer graphics; K. Yamamoto and W. D. Morgan for comments on the manuscript; M. Garlin for preparation of figures; and K. Erdley for typing the manuscript. This work was supported by grants from the NIH. D.G. held an EMBO fellowship and W.S.D. held a fellowship from the ACS, California Division Inc.

Received 3 September; accepted 26 September 1984.

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Isolation of cDNA clones encoding the 20K T3 glycoprotein of human T-cell receptor complex

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The complete amino acid sequence of one of the polypeptide chains of the human T3/T-cell receptor complex, the T3 glycoprotein (T3-δ-chain) of relative molecular mass 20,000, was deduced from cDNA clones derived from HPB-ALL cells and a human T-cell clone. Inspection of the 171-amino acid sequence reveals a signal peptide, a 79-amino acid extracellular domain, one transmembrane region and a 44-amino acid intracellular domain which show no sequence homology with members of the T-cell receptor/immunoglobulin/MHC multi-gene family. The T3 δ-chain is coded for by a single-copy gene the expression of which is restricted to T lymphocytes in humans and mice.

T LYMPHOCYTES have a central role in immune responses, eliminating cells that appear to be foreign and regulating the activities of other immune cells, including the antibody-producing B cells. Specific receptors are involved in antigen recognition by T-lymphocyte clones. Recently, it has been found that in humans and mice these T-cell receptors are sulphhydryl-linked heterodimers, which consist of two glycosylated polypep-

tide chains (α - and β -chains) of relative molecular mass (M_r) 37,000-50,000 (37K-50K)¹⁻³. In addition, genes encoding both the α - and β -chain have been isolated⁴⁻⁷. On the surface of human lymphocytes, the clone-specific (clonotypic) α - and β -chains of the T-cell receptor are associated with the three invariable T3 proteins, forming the so-called T3/T-cell receptor complex⁸⁻¹⁰. These T3 polypeptide chains have M_r of 25K (T3

Fig. 1 *a*, Oligonucleotide mixtures used as probes for cloning T3 δ -chain cDNA. Two regions of amino acid sequences from the N-terminal part of the T3 δ -chain were selected for synthesis of oligonucleotides. Probe 1 contained 576 different 19-mers with nucleotide sequences complementary to amino acid residues 2–8. Probe 2 contained 128 different 15-mers predicted from amino acid residues 12–16. Probe 1 represents (+) strand DNA and probe 2 (–) strand DNA. The oligonucleotide mixtures were chemically synthesized by the solid-phase phosphotriester method on an inorganic solid support and purified by HPLC as described previously^{34,35}. *b*, Result of second-strand synthesis using the 19-mer oligonucleotide mixture as primer. The ~0.62-kb cDNA product that is bracketed was used to generate the DNA library. O, Origin; XCF, xylene cyanole FF.

Methods: Total mRNA was extracted from HPB-ALL cells by lysis in a buffer containing 6 M guanidine hydrochloride³⁶. Poly(A)⁺ RNA was isolated by selective retention to oligo(dT) cellulose^{37,38}. 2 μ g of poly(A)⁺ RNA were converted to cDNA in the presence of 50 mM Tris-HCl pH 8.3, 1 mM each of the deoxynucleotide triphosphates (dNTPs), 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, 18 μ g ml⁻¹ of oligo(dT)₁₂₋₁₈, 40 U placental RNase inhibitor and 400 U ml⁻¹ reverse transcriptase (Life Sciences Inc.) in a final volume of 100 μ l. Incubation was carried out for 90 min at 42 °C. The reaction was stopped by the addition of EDTA to a final concentration of 20 mM and the mRNA template was removed by alkaline hydrolysis³⁸. After neutralization and phenol extraction, unincorporated dNTPs were removed by gel filtration through a 1-ml Sephadex G-50 spin column³⁸.

0.375 μ g of oligonucleotide probe 1 to be used as primer was 5'-end labelled with 100 μ Ci of [γ -³²P]ATP (5,000 Ci mmol⁻¹, ICN Radiochemicals) and 20 U T4 polynucleotide kinase (BRL). The reaction was stopped by adding EDTA to a final concentration of 20 mM. Following phenol extraction, the 5'-labelled oligonucleotide mixture was added to the cDNA and both were recovered by ethanol precipitation in the presence of 0.3 M NaAc, pH 5.5. The pellet was washed once in 70% ethanol and resuspended in 67 μ l of 104 mM KCl, 45 mM Tris-HCl pH 7.5 and 6 mM MgCl₂. The primer was annealed to the cDNA by heating for 5 min at 68 °C followed by slow cooling to room temperature. Before second-strand synthesis, the mixture was kept in ice for 1 h. Second-strand synthesis was initiated by the addition of dNTPs to a final concentration of 0.5 mM, 2-mercaptoethanol to a final concentration of 0.5 mM and 10 U DNA polymerase I (Klenow fraction). This mixture, in a final volume of 100 μ l, was incubated for 20 h at 15 °C, 20 U reverse transcriptase was added and incubation was continued for 1 h at 37 °C. The enzymes were inactivated by the addition of EDTA to a final concentration of 20 mM. Proteins were removed by phenol extraction and double-stranded (ds) cDNA was recovered by ethanol precipitation following gel-filtration through a 1-ml Sephadex G-50 spin column. The termini of the ds cDNA were trimmed with 30 U ml⁻¹ of S₁ nuclease in the presence of 0.3 M NaCl, 0.03 M NaAc pH 4.5 and 10 mM ZnCl₂ for 30 min at 37 °C in a final volume of 100 μ l. Following phenol extraction and ethanol precipitation, the cDNA was size-fractionated in a 5% polyacrylamide gel and a specific product of ~0.6 kb was detected after autoradiography. This product was excised from the gel and DNA was isolated and purified as described by Maxam and Gilbert²³. Homopolymeric dC tails were added using 20 U of terminal transferase (BRL) in the presence of 0.6 M sodium cacodylate pH 7.19, 0.5 mM dithiothreitol, 2.5 mM CoCl₂, 0.5 mg ml⁻¹ bovine serum albumin (BRL) and 5 μ M dCTP in a final volume of 50 μ l for 20 min at room temperature³⁹. After enzyme inactivation and phenol extraction, ds cDNA was recovered by ethanol precipitation, annealed to *Pst*I-digested, dGMP-tailed plasmid vector pBR322 and used to transform *E. coli* MC1061 (ref. 40) to tetracycline resistance. A selected cDNA library of ~7,000 members was generated by plating the cells directly onto nitrocellulose³⁵. After replica-plating and amplification, the DNA was denatured and fixed onto the filters as described elsewhere³⁸. The filters were hybridized overnight at 36 °C (probe 1) or 26 °C (probe 2) in 6 $\times$ SSC. (1 $\times$ SSC=0.15 M sodium chloride and 0.015 M sodium citrate). 1 $\times$ Denhardt's solution (0.02% each of Ficoll 400, bovine serum albumin and polyvinylpyrrolidone), 0.05% sodium pyrophosphate, 100 μ g ml⁻¹ of yeast tRNA and 10⁶ c.p.m. ml⁻¹ of 5'-³²P-labelled oligonucleotide probe 1 or 2 (specific activity ~4 $\times$ 10⁶ c.p.m. μ g⁻¹). Filters were washed extensively at room temperature in 6 $\times$ SSC plus 0.05% sodium pyrophosphate followed by stringent washes at 44 °C (probe 1) or 34 °C (probe 2) in the same buffer. The filters were exposed to Kodak XAR-5 film at -70 °C using an intensifying screen. One cDNA clone (pPGBC-1) with an insert of 0.62 kb which hybridized to both probes was identified.

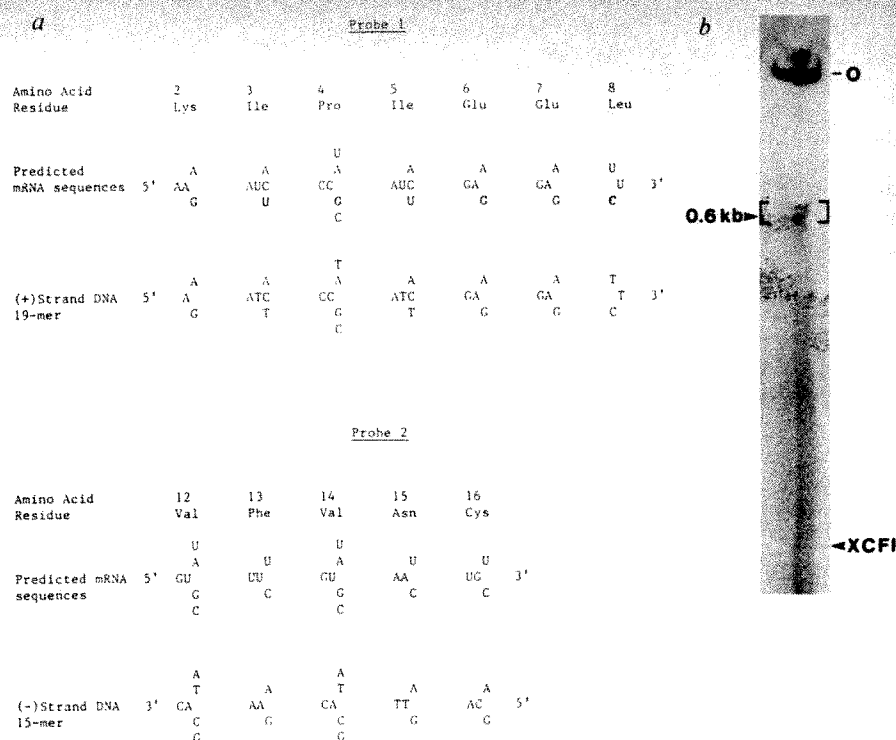
γ -chain) and 20K (T3 δ - and ϵ -chains). The T3 γ - and T3 δ -chains are glycoproteins whereas the T3 ϵ -chain contains no detectable oligosaccharides^{8,11-14}. The three T3 polypeptide chains are structurally unrelated to each other as determined by peptide mapping, N-terminal amino acid sequencing and their reactivity with an antiserum prepared using a synthetic peptide representing the 14 N-terminal amino acids of the T3 δ -chain¹⁵.

The T3 δ -chain is of particular interest because a monoclonal antibody that binds specifically to this glycoprotein is mitogenic for peripheral blood T lymphocytes¹⁶. This property had been ascribed earlier to other anti-T3 monoclonal antibodies, the exact target structure of which was unknown¹⁷⁻²⁰. When isolated from the plasma membrane, the T3 δ -chain has a polypeptide backbone of 14K after removal of N-linked oligosaccharides with endo- β -N-acetylglucosaminidase F (Endo-F)^{8,14}. In pulse-chase experiments using ³⁵S-L-methionine and ³⁵S-L-cysteine, a 23K intermediate was detected which was sensitive to endoglycosidase H (Endo-H) and Endo-F and which had a polypeptide backbone of 16K⁸. The N-terminal amino acid sequences of the 23K and 16K intermediates were identical to that of the 14K T3 (ref. 15). These observations indicated that both oligosaccharide and proteolytic processing might take place during the maturation of the T3 δ -chain. An alternative interpretation which postulated that the 16K product was an intermediate in the deglycosylation experiment could not be excluded, however.

Here we describe the isolation and characterization of cDNA clones that encode the complete amino acid sequence for the T3 δ -chain. The predicted *M_r* of the T3 δ -polypeptide chain is 16,719. This was confirmed by analysis on SDS-polyacrylamide gel electrophoresis of an *in vitro* translation product after hybrid selection. Taken together with our previous observations, these data provide strong evidence that proteolytic processing occurs at the C-terminus of the T3 δ -chain. Further inspection of the amino acid sequence revealed an 79-amino acid extracellular domain and a 44-amino acid intracellular domain separated by a transmembrane region of 27 amino acids. The amino acid sequence of this T3 chain shows no sequence homology with T-cell receptor α - and β -chains, immunoglobulins or T-cell surface structure Thy-1. T3 δ -chain mRNA was detected only in human haematopoietic cells of T-lineage. In murine helper T lymphocytes, a mRNA of similar size has been detected.

Isolation of cDNA clones

Plasmids with inserts coding for the T3 δ -chain were isolated from complementary DNA libraries using synthetic oligonucleotide mixtures as hybridization probes. From the available N-terminal amino acid sequence¹⁵, a 19-mer and a 15-mer oligonucleotide mixture was synthesized consisting respectively of 576 and 128 different sequences (Fig. 1*a*). First, in order to enrich for T3 δ -chain-specific sequences by size selection and to ascertain the presence of N-terminal protein coding sequences in the



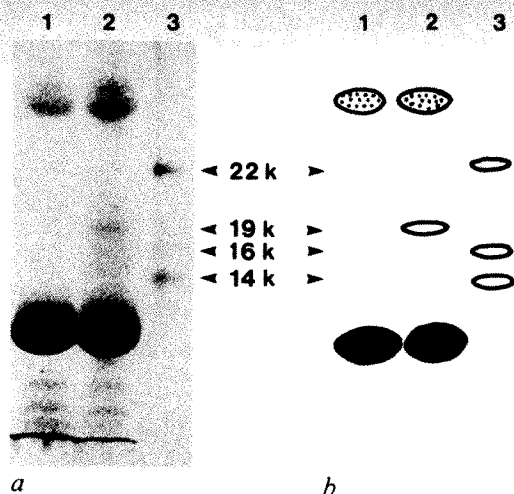


Fig. 4 SDS-polyacrylamide gel electrophoresis of the *in vitro* translation product of the T3 δ -chain mRNA. *a*, Lane 1, no RNA; lane 2, T3 δ -chain-selected mRNA; lane 3, Endo-F-treated anti-Leu-4 immunoprecipitate. *b*, Schematic representation of *a*. Numbers indicate the M_r values of the protein products discussed in the text.

Methods: 20 μ g of pPGBC-1, digested with *Pst*I, was spotted onto a nitrocellulose filter after denaturation. Poly(A)⁺ RNA was annealed to the bound T3 δ -chain cDNA in a buffer containing 70% formamide, 10 mM HEPES pH 6.8, 5 mM EDTA, 0.3 M NaCl and 0.2% SDS for 4 h at 47 °C. Following extensive washes with 1 $\times$ SSC, 0.5% SDS at 47 °C, the annealed RNA was released in a solution containing 90% formamide, 10 mM HEPES, 1 mM EDTA, 0.1% SDS and 25 μ g ml⁻¹ calf liver tRNA at 47 °C. T3 δ -chain-specific mRNA was isolated by ethanol precipitation in the presence of 0.2 M NH₄Ac, pH 5.5. The mRNA was translated in a rabbit reticulocyte system (BRL) in the presence of ³⁵S-L-methionine (Amersham). The products were electrophoresed in a 12.5% polyacrylamide SDS gel and compared with deglycosylated T3 proteins. For this preparation cells from the T-leukaemic cell line HPB-ALL were metabolically labelled with 1 mCi of ³⁵S-L-methionine and 1 mCi ³⁵S-L-cysteine (Amersham). The 20K proteins of the T3 complex were isolated by immunoprecipitation with the monoclonal antibody anti-Leu-4 as described previously⁸. The immunoprecipitate was subsequently treated with Endo-F as described elsewhere⁸. Following electrophoresis, the gel was treated with EN³Hance (NEN), dried and exposed to Kodak XAR-5 film at -70 °C.

peptide comprises a core of 19 hydrophobic amino acids which is flanked by two polar amino acids. Further analysis of the distribution of polar and nonpolar amino acids along the T3 δ -polypeptide chain (Fig. 3) reveals the presence of a hydrophobic segment (residues 80-106 in Fig. 2) which probably represents the transmembrane portion of the molecule. Although there is one aspartic acid residue (at position 90 in Fig. 2) in this stretch of hydrophobic amino acids, the negative charge of this amino acid does not influence the overall hydrophobic character of this stretch of amino acids (Fig. 3). The presence of charged amino acid residues in transmembrane segments has been observed in class I and class II antigens of the major histocompatibility complex (MHC) and T-cell receptor α - and β -chains^{4,6,7,24,25}. The unusual presence of a positively charged residue (Lys) in the T-cell receptor α - and β -chains suggests a possible salt bridge between one of these chains and the T3 δ -chain.

The first 79 N-terminal amino acids of the T3 δ -chain contain two potential sites for asparagine-linked oligosaccharides (residues 17 and 53, Fig. 2), in agreement with our previous Endo-H and Endo-F studies of the mature protein^{8,13}. The relative positions of the two oligosaccharide side chains and the transmembrane segment suggest that the 79 N-terminal amino acids are located on the extracellular side of the plasma membrane, and that residues 107-150 are intracellular. Interestingly, the intracellular sequence just C-terminal to the transmembrane

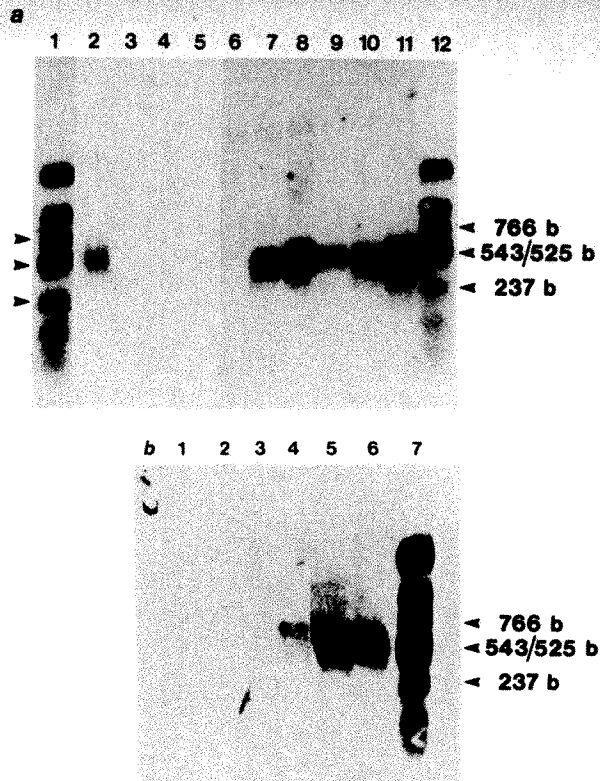
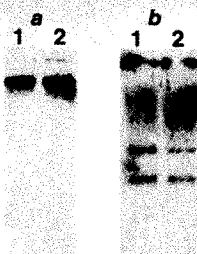


Fig. 5 Northern blot analysis of T-cell and non-T-cell RNA for the presence of T3 δ -chain specific mRNA. RNA from the following sources was used: *a*, Lanes 2, 8, human T-leukaemic cell line HPB-ALL; lane 3, HeLa cells; lane 4, human fetal brain; lane 5, human bone marrow; lane 6, human B-lymphoblastoid cell line JY; lane 7, human T-leukaemic cell line Molt-4; lane 9, T-leukaemic cells from a Sézary syndrome patient (T3⁺, T4⁺, T8⁺); lane 10, human thymus; lane 11, human T-leukaemic cell line JM-2. Lanes 1 and 12 represent a 5'-end-labelled *Hinf* digest of simian virus 40 DNA (SV40) DNA used as size markers. *b*, Lane 1, murine L-cells; lane 2, murine B-cell hybridoma A20; lane 3, murine T-T hybridoma (T-helper cell) B3C6; lane 4, murine T-T hybridoma (T-helper cell) B8C3; lane 5, human HPB-ALL cells; lane 6, human Molt-4 cells; lane 7, SV40 $\times$ *Hinf* size markers. Numbers on the right-hand side indicate the size (in bases) of some of the fragments of SV40 generated by *Hinf* digestion of 20 μ g of total RNA (*a*, lanes 2-5) or 4.5 μ g of poly(A)⁺ RNA (*a*, lanes 6-11, and *b*, lanes 1-6) were separated by electrophoresis in 1% agarose-formaldehyde gels³⁸ for 6 h at 5.5 V cm⁻¹. Following partial alkali hydrolysis, the RNA was transferred to nitrocellulose as described elsewhere³⁸. The blot was baked for 2 h at 80 °C, washed once in 3 $\times$ SSC, 0.2% SDS for 30 min at 65 °C and once in the same buffer for 30 min at 80 °C. The filters were prehybridized in a buffer containing 3 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.5% SDS, 0.05% sodium pyrophosphate and 100 μ g ml⁻¹ of denatured salmon sperm DNA for 4 h at 65 °C. Hybridization was performed for 16 h at 65 °C in a buffer containing 3 $\times$ SSC, 1 $\times$ Denhardt's solution, 0.05% sodium pyrophosphate, 100 μ g ml⁻¹ denatured salmon sperm DNA and 5 $\times$ 10⁶ c.p.m. ml⁻¹ pPGBC-1 cDNA insert, ³²P-labelled by nick-translation. Following hybridization, the filters were washed three times at room temperature in 3 $\times$ SSC, 0.1% SDS and twice at 65 °C in the same buffer for 15 min each. The blots were exposed to Kodak XAR-5 film at -70 °C using an intensifying screen.

region does not contain the stretch of positively charged amino acids frequently observed in other membrane proteins²⁵.

Comparisons between the nucleotide and protein sequences of the T3 δ -chain and the T-cell receptor α - and β -chains using the NUCALN and PRTALN programs show no strong homologies and no significant homologies between the T3 δ -chain and immunoglobulin polypeptide chains or rat and murine Thy-1. The latter glycoprotein has often been thought of as the murine T3 equivalent^{27,28}. Further searches using the Wilbur-Lipman search program for homologous protein sequences in

Fig. 6 Genomic representation of T3 δ -chain DNA from HPB-ALL cells or human thymus was used to analyse T3 δ -chain-specific sequences. Lanes 1, HPB-ALL DNA; lanes 2, thymus DNA. 10 μ g of cellular DNA was digested with *Eco*RI (a) or *Bam*HI (b). DNA fragments were separated in a 1% agarose slab gel and blotted onto nitrocellulose after denaturation of the DNA in the gel^{43,44}. T3 δ -chain-specific sequences were detected by hybridization with a ³²P-labelled T3 δ -chain cDNA insert of pPGBC-1.



the Dayhoff database were unsuccessful²⁹. The T3 δ -chain, unlike the α - and β -chains of the T-cell receptor, therefore seem not to belong to the immunoglobulin/MHC/T-cell-receptor multi-gene family.

Protein processing

The M_r of the protein backbone of the T3 δ -chain was estimated from the predicted amino acid sequence at 16,719, apparently larger than the 14K measured for the deglycosylated membrane bound T3 δ -chain. We therefore determined the size of the protein product of the T3 δ -chain mRNA following *in vitro* translation. Poly(A)⁺ mRNA from HPB-ALL cells was hybridized to clone pPGBC-1 immobilized onto a nitrocellulose filter. After removal of the unattached mRNA by several washes, the annealed mRNA was released and translated *in vitro* in a rabbit reticulocyte system. Figure 4 (lanes 2) shows that a 19K protein was synthesized from this mRNA and migrated considerably more slowly than the deglycosylated T3 δ -chains—the 14K and 16K products (lanes 3)—because the *in vitro* translation product contained the signal peptide (2.2K) which is not removed by proteolysis in the reticulocyte translation system. This demonstrated that the T3 δ -polypeptide chain indeed has a M_r of 16K. Because we consistently find a protein backbone of 14K on deglycosylation of the membrane-bound T3 δ -chain, a proteolytic step must have occurred to convert the 16K into the 14K backbone. As we have determined previously that the 16K product had an N-terminal sequence identical to the 14K species, it seems that a C-terminal fragment of the T3 δ -chain is removed by proteolysis.

Expression of T3 δ -chain mRNA

Monoclonal antibodies directed at the T3 complex (OKT3, Leu-4, UCHT1) have shown that it is present on all human peripheral blood thymocytes, medullary thymocytes and to a lesser extent on cortical thymocytes³⁰⁻³³. To examine expression of the T3 δ -chain mRNA, several haematopoietic and other cells were analysed by Northern blot analysis (Fig. 5). T3 δ -chain mRNA was detected in thymocytes, peripheral blood lymphocytes and T-leukaemic cells, but not in the B-lymphoblastoid cell line JY, bone marrow cells, HeLa cells or fetal brain tissue (Fig. 5a). This demonstrated that the T3 δ -chain mRNA is present in cells of T lineage only.

Studies with monoclonal antibodies directed at murine T lymphocytes have not resulted in the detection of a murine T3 equivalent. This is somewhat surprising in view of the strong structural similarity between the human and murine T-cell receptor α - and β -chains¹⁻⁶. Northern blot analysis using the insert of the cDNA clone pPGBC-1 as hybridization probe revealed

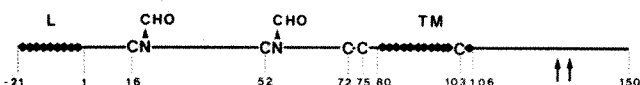


Fig. 7 A model for the T3 δ -chain. General features of the organization of the T3 δ -chain. CHO are the N-linked glycosylation sites. The arrows indicate the area of proteolytic processing. Stretches of hydrophobic amino acids are represented by the series of diamonds. L represents the leader sequence, TM the transmembrane region and C and N, cysteine and asparagine residues respectively. The numbers refer to the relative positions of the methionine initiator codon, the first amino acid of the mature protein, the termination codon, the position of the cysteine residues and the transmembrane region.

a 0.7-kb mRNA in the T-T hybridoma lines (T helper) B8C3 and B3C6 (Fig. 5b). In contrast, no obvious hybridization was observed with RNA from B-cell hybridoma A20 or from L-cells.

Taken together, the analysis of human and murine mRNAs shows that expression of the T3 δ -chain is restricted to T lymphocytes. Furthermore, the existence of a T3 δ -chain mRNA isolated from murine lymphocytes provides the first evidence that a murine T3 δ -chain equivalent exists.

Genomic representation of T3 δ -chain

Genomic DNA isolated from HPB-ALL cells and thymocytes was analysed by Southern blot analysis after digestion with *Eco*RI and *Bam*HI, respectively. The T3 δ -chain is located in both HPB-ALL cells and thymocytes on a single 13-kb *Eco*RI fragment (Fig. 6a). Digestion with *Bam*HI, which has a cleavage site in the deduced cDNA sequence at position 396, produces only two fragments of 3.3 and 2.2 kb (Fig. 6b). These findings indicate that the T3 δ -chain is represented as a single-copy gene in the genome.

Discussion

Several conclusions can be drawn from the amino acid sequence derived from cDNAs encoding the T3 δ -chain. The transmembrane segment consists of a stretch of 27 predominantly hydrophobic amino acids (residues 80–106 in Fig. 2) which is interrupted by an aspartic acid (residue 90 in Fig. 2). Asp 90 could form a salt bridge with the Lys residue in the transmembrane segment of the T3/T-cell receptor α - or β -chain^{4,6,7}. This type of bond would be pronounced due to the low dielectric constant of the hydrophobic environment. Unlike several other transmembrane segments, the T3 δ -chain hydrophobic domain does not contain a high level of α -helix-forming residues. Furthermore, this intracellular domain is not immediately followed by a string of positively charged amino acids, as in most transmembrane proteins²⁶. Perhaps association with the other transmembrane chains of the T3/T-cell receptor complex compensates for the need for such a positively charged area in the molecule. It is unlikely that the T3 δ -chain has a hairpin configuration, because protease K experiments with 'inside-out' microsomal vesicles showed that all components present in anti-T3 immunoprecipitates span the plasma membrane¹⁴. The T3- δ -chain can therefore be divided into two functional domains: an extracellular one of 79 amino acids, with two N-linked oligosaccharides, and a cytoplasmic domain of 44 amino acids (Fig. 7). This cytoplasmic domain is larger in relation to the extracellular compartment than are the T3/T-cell receptor α - and β -chains, perhaps reflecting the signal transferring function of the invariable T3 chains as opposed to the ligand binding function of the clonotypic T-cell receptor heterodimer.

The primary translation product of the T3 δ -chain has a M_r of 19K, including a 2.2K signal peptide. This observation, combined with our previous finding that on deglycosylation a 16K and a 14K product are formed, strongly suggests a proteolytic processing step. Indeed, pulse-chase experiments have shown that a 23K intermediate may be converted into one of the 20K T3 bands. This 23K species could be converted to a 16K protein backbone with Endo-H whereas the 20K species could be converted into the 14K protein⁸. In contrast, mature 20K T3, isolated from a membrane fraction was always converted into a 14K protein after treatment with Endo-F. As the 14K and 16K T3 fragments have the same N-terminal sequences, the proteolytic processing must have occurred in the C-terminal cytoplasmic portion of the T3 δ -chain (Fig. 7). In view of the role of T3 in T-cell activation, it is interesting to speculate that this proteolytic processing may be involved in the activation of T lymphocytes following the binding of antigen to the T-cell receptor.

The distribution of the half-cysteine residues is somewhat unusual in that two are directly N-terminal to each of the asparagine-linked oligosaccharides. Another cysteine lies in the transmembrane region, a characteristic of several of the MHC class II antigens²⁴. Protein fragmentation studies (J.B. and C.T., unpublished) suggest that intra-chain sulphhydryl bridges may

exist between some of the five half-cysteines, although it is not known how these bridges are arranged within the molecule. There is no evidence for their involvement in inter-chain disulphide bridges with the other chains of the T3 complex.

The gene for the T3 δ -chain seems to be a single-copy element expressed in all T lymphocytes. Furthermore, comparisons with other known protein sequences indicate that the T3 δ -chain is a unique polypeptide. Several groups have argued that T3 has an analogous role to Thy-1 in the murine T lymphocyte. Indeed, monoclonal and conventional anti-Thy-1 antibodies are mitogenic for murine T lymphocytes^{27,28}, as are anti-T3 reagents. However, the data presented here demonstrate that no sequence homology exists between the T3 δ -chain and Thy-1. In addition, whereas the Thy-1 antigen is expressed on neuronal cells, the T3 δ - and the ϵ -chains are not¹⁶. As the T3 ϵ -chain is not glycosylated and is probably a protein with multiple membrane segments, the only possible Thy-1-like member of the T3 complex is the 25K T3 γ -chain.

Our data demonstrate the existence of a murine equivalent of the human T3 δ -chain. The fact that this polypeptide chain has never been detected on the cell surface with murine antibodies specific for murine T lymphocytes may be due to its lack of genetic polymorphism. Indeed, comparison of human T3

δ -chains from different individuals by isoelectric focusing has not revealed any polymorphism in man¹².

The structural studies described here provide a basis from which to study the biosynthesis, surface expression and function of the T3 complex in a variety of different lymphocyte clones, and during T-cell differentiation. The extent of association between the T3 δ -chain and the clonotypic heterodimer is obviously determined by the properties of all five components of the T3/T-cell receptor, and this association may in fact govern the functional response following the binding of antigens to its receptor. In such a model, the T3 δ -chain may be involved in transducing a signal to the intracellular compartment.

We thank Dr Ken-Ichi Arai (DNAX, Palo Alto) for a cDNA library prepared with RNA from a human T-cell clone; Drs Rachel Neve and Linda Clayton for generous gifts of RNA preparations; Dr Alan Maxam and Karen Gruskin for advice in computer aided sequence analysis; and Sharon Bucchini for manuscript preparation. This work was supported by grants from the NIH (AI-15066 and AI-17561). P.v.d.E. was supported by fellowships from the Netherlands Organization for the Advancement of Pure Research (ZWO/NATO Science Fellowship) and the Niels Stensen Stichting. C.T. is a scholar of the Leukemia Society of America.

Received 13 September; accepted 20 September 1984.

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Novel immunoglobulin heavy chains are produced from DJ_H gene segment rearrangements in lymphoid cells

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It has been found that most immunoglobulin heavy(H)-chain gene diversity (D) segments carry their own 5' transcriptional promoter element. Transcription of rearrangements which use heavy-chain D and joining (J) segments in B-lymphoid cells ultimately leads to the production of a $D\mu$ messenger RNA which contains the DJ_H segments linked to the μ heavy-chain constant region. If the D is joined to the J_H segment in the appropriate translational reading frame, this $D\mu$ mRNA is translated to yield a short $D\mu$ protein with a variable DJ_H N-terminus.

THE variable region of a classical immunoglobulin heavy-chain gene is assembled from three germ-line components: the variable (V_H) segment, the diversity (D) segment and the joining (J_H) segment¹⁻³. These segments lie upstream of the constant (C_μ) region. The D segment has an important role in this somatic gene assembly process by mediating the joining of V_H and J_H

elements. This joining process is ordered: a D element combines first with a J_H element to form a DJ_H complex to which a V_H element is subsequently appended⁴. In the expressed V_H domain, the D sequence encodes part of the third complementarity-determining region and is thus important in generating antibody diversity⁵. Twelve D elements, 1-80 kilobases (kb) upstream

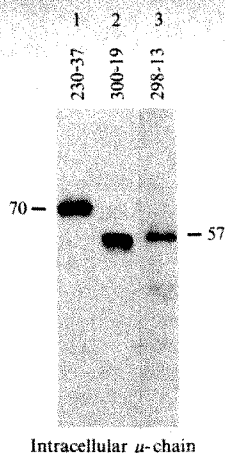


Fig. 1 Expression of truncated heavy-chain gene products in A-MuLV transformants. Approximately 10^7 cells were lysed in phospholysis buffer³⁴, the lysates reduced in the presence of mercaptoethanol, fractionated by electrophoresis through 10% polyacrylamide gels³⁵ and electrotransferred to a nitrocellulose filter for 4 h at 150 V in a Transblotter (Biorad). Nonspecific binding sites on the filter were saturated by a 5-h incubation in a 10% casein/phosphate-buffered saline (PBS) solution and the bound μ chains detected by a sandwich technique using a goat anti-IgM antiserum (a gift of A. Radbruch) and ^{125} I-labelled monoclonal IgM antibody B1-8 (ref. 36).

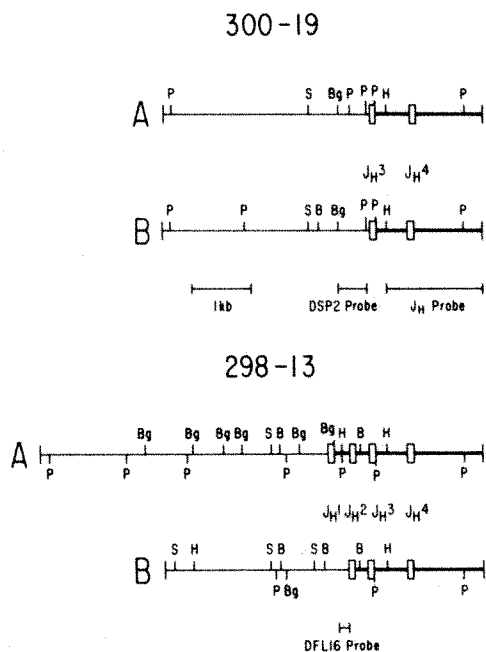


Fig. 2 Restriction maps of cloned DJ_H alleles of 300-19 and 298-13. The two DJ_H rearrangements in each of these lines were arbitrarily designated as A or B. Individual rearrangements were isolated by cloning complete *Eco*RI-digested genomic DNA from the appropriate lines into Charon 16a and screening the resulting libraries for recombinants which hybridized to a J_H probe as described previously⁹. Inserts from the appropriate recombinant phage were subcloned into PUC-9 and partial restriction maps constructed by single, double or triple digestions of the plasmid DNA with the indicated restriction enzymes (B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; P, *Pst*I; S, *Sac*I). The J_H part of the DJ_H complex is indicated by a darker line. The location of the J_H , DSP2 and DFL16 probes are indicated below the restriction maps; the DSP2 probe is a 500 bp *Bgl*II-*Bst*EII fragment from the DSP2 J_H rearrangement of P4-12 (see Table 1) which had a *Bst*EII site at the DJ_H joint. The DFL15 probe is a 100 bp *Ava*II-*Sau*3A fragment, spanning the region -120 to -10 in Fig. 3a.

from the J_H locus, have been described in the mouse genome⁶. These elements comprise three families (5' to 3'), DFL16 (two members), DSP2 (nine members) and DQ52 (one member)⁷.

Transformation of bone marrow or fetal calf liver cells with Abelson murine leukaemia virus (A-MuLV) generates permanent lymphoid cell lines. Most transformants represent the pre-B stage of B-lymphocyte differentiation and provide a model system for the study of the early stages of immunoglobulin gene rearrangement and expression⁸. Some A-MuLV-generated lines produce an unusually small μ heavy-chain messenger RNA and sometimes a small μ protein⁹. We report here that the short μ mRNA sequences arise from the transcription of DJ_H rearrangements and the short μ proteins from the translation of the resulting $DJ_H C_\mu$ -containing mRNAs ($D\mu$ mRNA). Because most of the D segments have the potential for expression after juxtaposition to a J_H segment, we suggest that they may have a function independent of their association with a V_H gene segment.

A short μ -chain in A-MuLV transformants

We studied two lines which produce short μ -chains, 300-19 and 298-13 (ref. 10), derived from the bone marrow of NIH/Swiss and BALB/c mice, respectively. The size of these μ -chains was determined by fractionating cell lysates from each line by electrophoresis through SDS-polyacrylamide gels, transferring to nitrocellulose paper¹⁰ and detecting immunoglobulin chains by a sandwich technique using anti-IgM antisera and ^{125} I-labelled monoclonal IgM antibody. We also analysed lysates from a control transformant (230-37) which produces normal-sized μ -chains of molecular weight $\sim 70,000$ (70K) (Fig. 1, lane 1). Both the 300-19 and 298-13 lines clearly contained abnormally small μ proteins of approximately 57K (Fig. 1, lanes 2, 3). Furthermore, we confirmed that instead of the normal 2.4 and 2.7 kb μ mRNAs which encode, respectively, the secreted and membrane-bound forms of the μ protein^{11,12}, the 300-19 and 298-13 lines contain truncated C_μ -specific RNAs of 2.0 and 2.3 kb; these species contain 3' ends specific to the membrane and secreted forms of the protein, respectively⁹. We suggest that the truncated μ -chains are encoded by correspondingly truncated μ mRNA species.

Production of the short μ -chain

Rearrangements of the J_H alleles in the 300-19 and 298-13 lines were detected and characterized by genomic DNA blotting assays using D - and J_H -specific probes as described previously⁴. These analyses indicated that each line had DJ_H rearrangements at both J_H alleles (data not shown) and thus represented a very early stage of B-cell differentiation⁴. To elucidate these rearrangements further, we cloned *Eco*RI-digested genomic DNA from the two lines into λ phage Charon 16A and isolated recombinants which hybridized to a J_H -specific probe. We show the restriction maps of the cloned DJ_H alleles (denoted A and B) of each line (Fig. 2) and the nucleotide sequence around the DJ_H joints (Fig. 3a). These data indicate that both DJ_H rearrangements of the 300-19 line involved a J_H3 segment and similar yet distinct DSP2 elements, whereas the DJ_H rearrangements on the A and B allele of the 298-13 line involved joining of DSP2 to J_H1 and DFL16.1 to J_H2 , respectively. All four of these D elements carried the conserved recognition heptamer and nonamer sequences separated by the characteristic 12 base pair (bp) spacer². The three DSP2 segments have a very similar 5' flanking sequence which differed from DFL16 in over 30% of the nucleotide positions. Each DJ_H joint occurred in different positions of the D or J_H germ-line sequence, indicating the flexibility of the joining process^{13,14}. For example, on allele A of 300-19 (Fig. 3a), the rearrangement event resulted in the deletion of most of the DSP2 coding sequence and a large part of the J_H sequence. This allele also had a 12-nucleotide sequence between the D and J_H segments which was not present in the germ-line sequence of either element; such 'N-region' sequences (cross-hatching in Fig. 3) are thought to be generated *de novo*

Table 1 Expression of $D\mu$ RNA and $D\mu$ chain in lymphoid cell lines and tissues

Cell line	Rearrangements		DSP RNA	DFL RNA	$D\mu$ protein
	Allele A	Allele B			
Bone marrow					
300-19	DSPJ _H 3	DSPJ _H 3	+	-	+
300-19P3	—	DSPJ _H 3	+	-	+
300-19P18-7-7	DSPJ _H 3	—	+	-	-
300-19P8	VDJ _H 3	VDJ _H 3	-	-	-
298-13	DSPJ _H 1	DFLJ _H 2	+	+	+
298-13-9	VDJ _H 3	DFLJ _H 2	-	+	+
P4-12	DSPJ _H 4*	DFLJ _H 4*	+	+	-
18-48	DFLJ _H	—	-	+	-
1-8	VDJ _H	DFLJ _H	-	ND	-
3-1	VDJ _H	VDJ _H	-	ND	-
298-8	VDJ _H	VDJ _H	-	ND	-
230-37	VDJ _H	VDJ _H	-	ND	-
Fetal liver					
22D6.10	DFLJ _H 4*	DSPJ _H 3*	+	ND	-
22D6.C11	DFLJ _H 4	DSPJ _H 4	+	ND	+
22D7	DSPJ _H	DFLJ _H	+	ND	-
38B11	DFLJ _H	DSPJ _H	+	ND	-
38B9	DFLJ _H *	DSPJ _H	+	ND	-
40E1	DSPJ _H	DSPJ _H	+	ND	-
40E4	DSPJ _H *	DSPJ _H *	+	ND	-
40E3	DFLJ _H	DFLJ _H	-	ND	-
41B1	DFLJ _H	DFLJ _H	-	ND	-
38B7	VDJ _H	VDJ _H	-	ND	-
Myeloma					
J606	DSPJ _H	VDJ _H	+	ND	ND
H2020	VDJ _H	VDJ _H	-	ND	ND
MPC11	VDJ _H	VDJ _H	-	ND	ND
M315	VDJ _H	VDJ _H	-	ND	ND
T-cell lines					
TB1	DSPJ _H	—	+	ND	-
TB2	DJ _H	DJ _H	+	ND	-
TB3	DJ _H	DJ _H	+	ND	ND
Tissue					
15-Day fetal liver			+	ND	ND
21-Day fetal liver			+	ND	ND
Adult spleen			+	ND	ND
21-Day thymus			+	+	ND
Adult thymus			+	+	ND

The rearrangement status of the J_H alleles (A and B) in A-MuLV transformants and myeloma lines were analysed by Southern blotting, using D - and J_H -specific probes as described previously⁴. The presence of $D\mu$ RNA in various cell lines and tissues was detected by a slot blot procedure. Briefly, 5 μ g of total RNA or 0.5 μ g of poly(A)-containing RNA was denatured in 6% formaldehyde, 50% formamide and 1×SSC at 68°C for 10 min, diluted 4× with 20×SSC and then blotted onto nitrocellulose filters using a slot-blotting apparatus (Schleicher & Schull, minifold 2). The filters were then baked, incubated with DSP2- or DFL16-specific probes (see Fig. 2 for description of probes) and washed as described previously⁹. The presence of the $D\mu$ chain was analysed by Western blotting as described in Fig. 1 legend. The presence or absence of $D\mu$ RNA and $D\mu$ chain is indicated by + and - in the appropriate column. Deletion of one of the J_H alleles in certain lines was defined by the presence of only one J_H -positive band in a Southern blot of genomic DNA cut by a panel of restriction enzymes and is indicated by a - in the rearrangement column. ND, not done.

* These J_H alleles were cloned, sequenced and proven to have an out-of-phase DJ_H joint as described in the text (ref. 33 and M.G.R. and F.W.A., in preparation).

during the D to J_H joining process¹⁵. N regions were also present in two of the other DJ_H rearrangements and contributed significantly to the variability of the DJ_H complex (Fig. 3a). These sequencing analyses demonstrated unequivocally that both J_H rearrangements of 300-19 and 298-13 were DJ_H joints and suggested that the short μ transcripts and proteins in these lines were derived from a DJ_H rearrangement. We will now refer to these products as $D\mu$ mRNA and $D\mu$ protein.

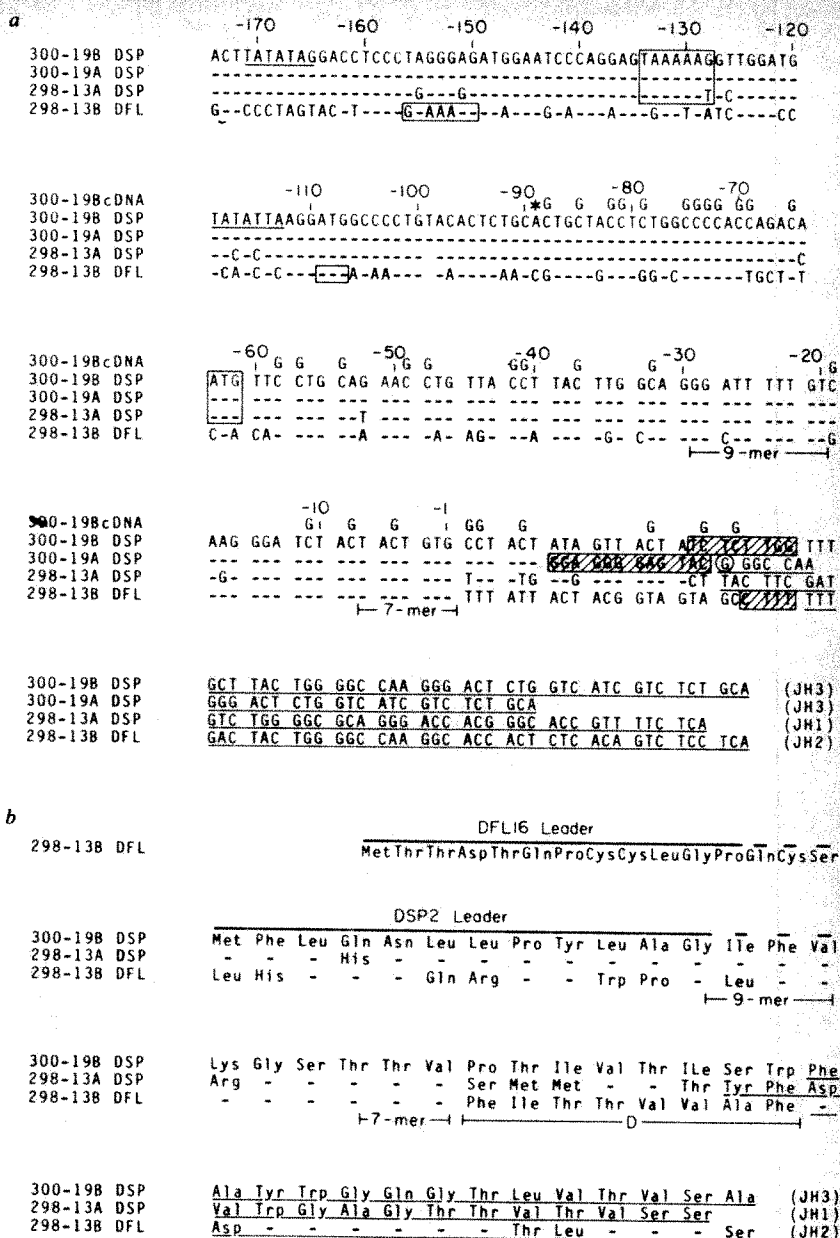
We next determined whether the $D\mu$ chain was encoded by one or both DJ_H alleles in 300-19 and 298-13. This analysis was possible because both lines, like fetal liver-derived A-MuLV transformants with two DJ_H rearrangements⁴, undergo 'secondary' rearrangements of their DJ_H complexes during propagation in culture (M.G.R. and F.W.A., in preparation). These secondary rearrangement events include appendage of V_H segments to the DJ_H complexes with the accompanying deletion of all sequence lying between the joined V_H and DJ_H segments⁴ and non- V_H -mediated deletion events which result in the complete loss of one of the DJ_H alleles as well as the flanking D and J_H segments. The nature of each J_H -specific secondary

rearrangement in a collection of 300-19 and 298-13 subclones was determined by the Southern blotting assay described previously⁴ and compared with μ -chain expression in the respective lines (Fig. 4). Digestion of total cellular DNA simultaneously with *Bam*HI and *Eco*RI liberates the DJ_H A and B alleles of the 300-19 parent line as distinguishable J_H -specific fragments of 5.5 and 2.8 kb (Fig. 4A, lanes 1; see Fig. 2 for description of J_H probe and maps of rearrangements). Both fragments are clearly different from the 2.1 kb unrearranged, J_H -positive fragment observed in *Bam*HI-*Eco*RI-digested liver DNA (Fig. 4A, lanes labelled L).

Analysis of two subclones of the 300-19 line which, because of non- V_H -mediated deletion events, contained only the DJ_H A allele (P18-7-7) or the DJ_H B allele (P3), demonstrated that only the line containing the latter allele produced the $D\mu$ chain (Fig. 4A, lanes 2, 3; compare a and b). This result indicates that the $D\mu$ chain in 300-19 is derived from the DJ_H B but not the DJ_H A rearrangement (although both DJ_H alleles transcribe a $D\mu$ mRNA). A study of 300-19 daughter cells which underwent V_H to DJ_H rearrangement during propagation in culture added

Fig. 3 a. The nucleotide sequence of the 300-19 and 298-13 *DJ_H* rearrangements. The *DSP2J_H3* sequence of the 300-19 B allele is given as the reference sequence, and identity of the other *DJ_H* sequences, is indicated by a dash. The 5' flanking sequence of the *D* segments is marked by negative numbers starting from the beginning of the heptamer. The free space at position -99 indicates a one-nucleotide deletion in the 298-13 sequences relative to the 300-19 sequences. The sequences are divided into codons according to the predicted *DSP2* and known *J_H* reading frames. The circled G nucleotide at the *DJ_H* joint of the 300-19 A allele indicates that the ATG of the *D* segment is not in the same translational reading frame as the *J_H* coding sequence. Promoter-like elements and the presumed ATG start codons of the SP2 and FL16 *D* segments are boxed. Other TATA-box-like elements are underlined. *N*-region sequences are symbolized by hatched boxes, and the *J_H*-derived sequences are underlined. The probable cap site of the *DSP2μ* mRNA derived from allele B of 300-19 (deduced from the primer extension studies) is indicated by an asterisk; the G-track of the 5' portion of the 300-19 B allele cDNA sequence is shown above the corresponding DNA sequence. **b.** Sequence of the variable portion of the *Dμ* chain. The amino acid sequence of the expressed (in phase) *DJ_H* alleles was predicted from the nucleotide sequences in **a**. The position of the nonamer, heptamer, traditional *D* coding region, and the *J_H* sequences are indicated. The presumed leader peptides of *DFL16* and *DSP2* are overlaid.

Methods: The nucleotide sequence of the four cloned *DJ_H* joints was determined by the method of Maxam and Gilbert³⁷. The transcriptional initiation site of the *DSP2μ* mRNA was determined by primer extension methods^{38,39}. A 15 bp-long *Cμ* 1 primer (a gift of E. Kabat) was kinased with ³²P, annealed to total poly(A)⁺ RNA from the 300-19P3 subclone and extended by reverse transcription as described previously¹¹. Primer extended cDNA was fractionated by 4% polyacrylamide gel electrophoresis and the major elongation product (a 173 bp cDNA) was isolated by electroelution.



further support for this conclusion: *V_HDJ_H* rearrangements can be classified as either 'productive' or 'non-productive' depending on whether or not they lead to the production of a normal heavy-chain protein^{4,9}. Productive appendage of a *V_H* segment to the *DJ_H* B allele in subclone P14 (2.5 kb *J_H*-positive band; Fig. 4A, lane 6) led to the replacement of the *Dμ* protein by a normal-sized *μ* chain (Fig. 4B, lane 6). Non-productive rearrangement of both *DJ_H* alleles in subclone P17 generated two new *J_H*-positive bands of 2.1 and 1.9 kb and resulted in the loss of any *μ*-chain production by the line (Fig. 4A, lane 5). Subclone C15, on the other hand, which underwent a productive *V_H* to *DJ_H* rearrangement on the A allele and left the *DJ_H* B allele intact (Fig. 4A, lane 4), did not lose *Dμ* expression, but instead produced both the truncated and normal-sized *μ* chains simultaneously (Fig. 4B, lane 4).

Secondary rearrangements of the A and B alleles in subclones of the 298-13 line were analysed similarly. The *DSP2* and *DFL16* to *J_H* rearrangements of the 298-13 parental line appeared as rearranged, *J_H*-positive *EcoRI* fragments of 7.8 (A) and 5.6 kb (B), respectively (Fig. 4Ab, lane 1; maps in Fig. 2). The 6.2 kb unrearranged *J_H*-positive *EcoRI* fragment of liver DNA is shown for comparison (Fig. 4Ab, lane L). A subclone of 298-13 with an aberrant *V_H* to *DSP2J_H* rearrangement (Fig.

4Ab, lane 2; 2.0 kb *J_H*-positive band) but retaining the intact *DFL16J_H* B allele continued to produce a *Dμ* chain (Fig. 4Bb, compare lanes 1 and 2). This demonstrates that *DFL16* elements also are capable of expression after joining to a *J_H* segment. Similar analyses of other subclones indicated that the *DSP2J_H* rearrangement of the 298-13 line also encoded a truncated *μ* chain (data not shown); thus both *DJ_H* alleles of the 298-13 line were expressed.

Initiation sites of *Dμ* mRNA and *Dμ* protein

The transcriptional initiation site of the *DSP2μ* mRNA was determined for the *DJ_H* B allele of 300-19 by primer extension studies. Poly(A)⁺ RNA from 300-19P3 cells (in which allele A is deleted, see above) was hybridized to a radioactive, 15 bp single-stranded DNA primer complementary to the nucleotide sequence 3-18 of the *Cμ* 1 exon. The bound primer was extended by reverse transcription and the resulting cDNA analysed by electrophoresis through a 15% polyacrylamide gel. The major elongation product was a cDNA of 173 nucleotides (data not shown). The nucleotide sequence of the G-track of this cDNA was determined (Fig. 3a). Both the size and the sequence of this cDNA defined the cap site of the *DSP2μ* mRNA as an

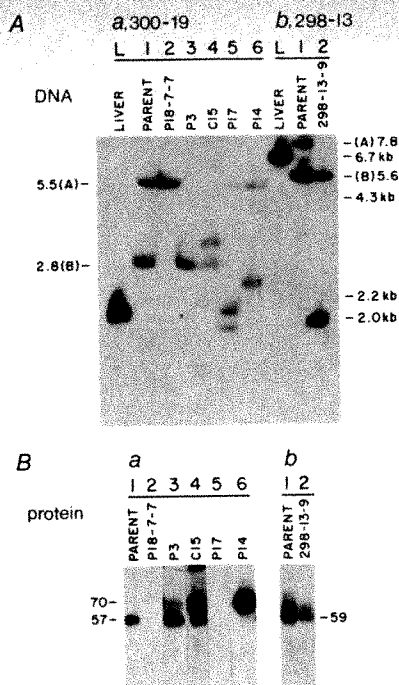


Fig. 4 J_H rearrangements and intracellular μ chains in subclones of 300-19 and 298-13 lines. Subclones of the 300-19 and 298-13 lines were isolated by limiting dilution as described previously⁸. Deletion of or V_H appendage to the pre-existing DJ_H rearrangements of these lines was determined by the genomic blotting assays described previously⁴. **A**, DNA: Approximately 10 μ g of genomic DNA was digested either with a combination of the restriction enzymes *Bam*HI and *Eco*RI (**a**) or with *Eco*RI alone (**b**), electrophoresed through 1% agarose, blotted onto nitrocellulose and assayed for hybridization to a ³²P-labelled J_H probe (see Fig. 2). The bands corresponding to the two parental DJ_H alleles in each line are indicated as A and B, respectively. Other lanes contained DNA from individual subclones with either a deletion or a V_H to DJ_H rearrangement of the parental DJ_H alleles as described in the text. **B**, Protein: The cell lysate of each line analysed was fractionated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and bound μ heavy chains detected by ¹²⁵I-labelled antibodies as described in Fig. 1 legend.

adenine residue situated 89 nucleotides upstream of the D heptamer (position -89, asterisk in Fig. 3a). The promoter-like sequences TAAAAAG (300-19, both alleles) and TAAAAAT (298-13, allele A) were found 45 nucleotides upstream of the $DSP2$ transcriptional initiation site. Although the 300-19 $DSP2$ sequence contains two other TATA box-like elements (underlined in Fig. 3a), we regard the above sequences as the most likely $DSP2$ promoter, because we also found similar elements (GAAAAAG and TAAAAAG) in the 5' sequence of the FL16- and Q52-type D segments. Both of the latter D elements are transcriptionally active in cells which carry the respective DJ_H rearrangement, such as DFL16 (Table 1) and DQ52¹⁶. The 2.3 and 2.0 kb μ -chain-specific RNA sequences in 300-19 and 298-13 contain the four C_μ coding exons plus either a μ_m or μ_s 3' terminus (where m and s represent membrane-bound and secreted forms), respectively; these mRNA sizes are consistent with RNA transcripts initiated at the indicated D promoter elements and subsequently spliced to yield a 5' DJ_HC_μ 3' RNA sequence ($D\mu$ mRNA). In support of this conclusion, we find that the 2.3 and 2.0 kb C_μ -specific RNA sequences in these lines hybridize to probes specific for the putative D portion of the transcript (data not shown).

Analysis of the 5' $DSP2$ sequence revealed an ATG codon 26 nucleotides downstream of the transcriptional initiation site (position -63 in Fig. 3a) which is the beginning of an open reading frame throughout the following D sequence. Further-

more, the $DSP2J_H$ joint of allele B in 300-19 as well as the $DSP2J_H$ rearrangement of 298-13 (each of which were linked to the short μ -chain product, as described above) placed this ATG in phase with the known translational reading frame of the J_H coding sequence to which it was appended. The $DSP2J_H$ joint of allele A of 300-19, on the other hand (which does not encode a $D\mu$ chain), places the -63 ATG of the $DSP2$ element in a different translational reading frame from that of the linked J_H coding sequence (Fig. 3a). Additional studies with several other subclones and independent cell lines which contained $DSP2$ rearrangements indicated that only those which had the -63 ATG sequence in the same translational reading frame as the linked J_H sequence produced the $D\mu$ protein, although all produced $DSP2\mu$ RNA sequences (Table 1).

An ATG codon is not found at the -63 position in the DFL16 sequence; an ATG codon situation 48 nucleotides farther upstream (at position -109), however, divides the following D sequence into the same translational reading frame as does the $DSP2$ ATG at -63. In the 298-13 productive DFL16 J_H rearrangement, this ATG is in the same translational reading frame as the J_H coding region. In several other lines which had DFL16 J_H rearrangements but which did not produce $D\mu$ chains, the DFL16 ATG was out of phase with the J_H coding sequence (Table 1). We conclude that the $DSP2$ ATG at -63 and the DFL16 ATG at -109 are the translation initiation sites of the $DSP2$ - and DFL16-type $D\mu$ chain, respectively. These ATG sequences represent the first (most 5'-proximal) translation initiation codons on the respective $D\mu$ mRNAs and both ATG codons are flanked by 5' sequences (a conserved A or G residue three nucleotides upstream) that are important for efficient utilization¹⁷, which supports our conclusions.

Expression of $D\mu$ RNA and $D\mu$ protein

We used probes for SP2- and FL16-type D elements derived from the body of D and the immediate 5' flanking region to detect $D\mu$ mRNA in various B-lymphoid cell lines (Fig. 2). We find a strict correlation between the presence of the $D\mu$ mRNA and a DJ_H rearrangement (Table 1). $DSP2\mu$ mRNA is found in subclones of 300-19 (P3 and P18-7-7) with a deletion of either the A or the B allele, which proves that each 300-19 DJ_H allele is transcriptionally active. In 300-19 subclone P8, appendage of V_H segments to the DJ_H rearrangements of both alleles resulted in deletion of all D promoter sequences and $D\mu$ mRNA transcripts are no longer produced (Table 1). The 298-13 parent line produces both $DSP2$ - and DFL16-type μ chains, thus it contains both $DSP2$ - and DFL16-type $D\mu$ mRNA. A subclone (298-13-9) with a non-productive rearrangement of the $DSP2$ allele lost the $DSP2$ transcript but retained the FL16-type mRNA. Analysis of many different lines consistently showed that transformants with V_HDJ_H rearrangements at both J_H alleles (mostly of adult marrow origin) did not produce $D\mu$ mRNA/sequences, whereas transformants with one or more DJ_H rearrangements (mostly of fetal liver origin) always produced $D\mu$ mRNA (Table 1). Many of the lines analysed contained $D\mu$ mRNA but did not produce the $D\mu$ protein. Both J_H allele from three such lines were cloned, sequenced and proven to have an out-of-phase (non-productive) D to J_H joint with respect to putative 5' D translation initiation codons (Table 1).

We included four myeloma cells (representing terminally differentiated B cells) in the RNA analysis and found that the one line (J606) containing a $DSP2J_H$ rearrangement⁴ also produced $D\mu$ mRNA (Table 1). T-lymphoid cells frequently contain DJ_H rearrangements¹⁸⁻²⁰; we have found $D\mu$ mRNA in three T-cell TB lines²¹ that have these rearrangements and in RNAs extracted from normal lymphoid tissues such as adult spleen, fetal liver and newborn thymus (Table 1). The expression of $D\mu$ mRNA in spleen and fetal liver is consistent with our data derived from the various B-cell lines. The detection of D message in the newborn thymus, where contamination by B lymphoid cells should be negligible⁹, agrees with the previous finding of significant quantities of 2.3 and 2.0 kb C_μ -contain-

RNA sequences in this tissue^{9,22,23}, suggesting that some normal T-lymphoid cells produce $D\mu$ mRNA sequences.

Function of $D\mu$ chains

We have demonstrated that most D elements have a 5' transcriptional promoter. Like the V_H promoter, the D promoter is presumably activated if a DJ_H rearrangement places it in close proximity to the IgH enhancer element^{24,25,40,41}. When the DJ_H joint places the 5' D translation initiation codon in the same translational reading frame as the J_H coding sequence, the $D\mu$ mRNA encodes a short μ -protein. The definition of D segments as mini-genes²⁶ raises the question of what function the short $D\mu$ chain may have. The first 18 amino acids of the DSP2-derived μ -protein sequence have a striking similarity to known leader peptides²⁷, containing a stretch of 10 hydrophobic residues flanked by charged amino acids (Fig. 3b). We conclude that the DSP2 μ chain is inserted into the endoplasmic reticulum, supported by the observed glycosylation of this chain⁹. Although the $D\mu$ chain lacks a classical variable-region domain, it has a variable DJ_H peptide followed by the four μ -chain constant region domains and a membrane or secreted C-terminus. The DFL16-derived $D\mu$ protein differs from the DSP2 μ chain only in the N-terminus, which is 15 amino acids longer and starts with a different leader-like sequence (Fig. 3b). The structural consequence of this difference is that the hypervariable region of the DSP2 μ chain is close to the N-terminus whereas in the DFL16 μ chain it is flanked on the N-terminus by conserved D sequences. RNA from the 300-19 line (which only produces DSP2 μ chains) programmes synthesis of two distinct μ chains of 52K and 55K molecular weight whereas RNA from the 298-13 line (which produces both DSP2 μ and DFL16 μ chains) programmes four μ -chain translation products of 52K, 55K, 54K and 57K⁹. The translation products of the normal membrane and secreted forms of μ -chain mRNA are 67K and 64K, respectively^{11,12}; the 52K and 55K μ chains presumably represent the products of the membrane and secreted forms of DSP2 μ mRNA while the 54K and 57K chains represent those of the corresponding forms of DFL16 μ mRNA. The expression of the $D\mu$ protein is not subject to allelic exclusion in the 298-13 line, which supports the proposal that V_H to DJ_H joining is the allelically excluded step in V_H gene assembly^{4,9}.

$D\mu$ proteins can be expressed in conjunction with a C-terminus specific for the membrane form of the constant region, suggesting that they may be part of a novel, variable receptor system; however, we have not yet found the protein expressed on the surface of A-MuLV transformants. The normal-sized μ chain is found on 300-19 surfaces only with an immunoglobulin light chain (M.G.R. and F.W.A., in preparation), which suggests that a second chain may be necessary for surface expression. The detection of $D\mu$ transcripts in neonatal thymus (Table 1) may explain the presence of immunoglobulin-like chains on the surface of T cells^{28,29}. DJ_H rearrangements in T cells are generally thought to have no function³; our suggestion that they may encode a $D\mu$ protein warrants a re-evaluation of this idea. Surface $D\mu$ -chain expression could also contribute to the observed idiotypic cross-reaction between T and B cells³⁰.

Sequence variations between D elements expressed in the mouse strains used in our study maintained the positive charge of the putative leader peptide and did not impair $D\mu$ -chain expression (Fig. 3b). A sequence comparison of homologous murine and human DQ52 segments shows only a few stretches of homology³¹, one of which contains the D promoter-like sequences. Another human D element (D36)³¹ contains 5' promoter and ATG sequences in positions similar to the mouse DSP2 segment, although its expression is probably prevented by an in-phase translation stop codon. However, a short μ chain similar to the murine $D\mu$ chain has been found in human pre-B-cell lines derived from patients with X-linked agammaglobulinaemia, possibly as a result of incomplete V_H to DJ_H joining⁴². This evolutionary conservation of $D\mu$ -chain expression also implies a functional significance for this chain. D segments of the β chain of the T-cell receptor are also transcribed after the formation of a DJ_β rearrangement; the 5' flanking region of these segments also has potential translation initiation sites³². Thus the T-cell DJ_β rearrangements similarly may lead to the production of DJ_β polypeptide chains.

We thank E. A. Kabat, A. Radbruch, G. D. Yancopoulos, N. Rosenberg and K. Karjalainen for advice and assistance, and T. K. Blackwell and N. E. Kohl for critically reading the manuscript. This work was supported by ACS grant NP-393, NIH grant AI-20047 and a Searle Scholars award. M.G.R. was a recipient of a fellowship from the Deutsche Forschungsgemeinschaft and F.W.A. is an I. T. Hirschl Career Scientist.

Received 4 August; accepted 20 September 1984.

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Regulation of actin polymerization by non-polymerizable actin-like proteins

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Three functionally distinct actin-capping proteins from the slime mould Physarum are structurally closely related to actin itself. In Physarum, actin polymerization is regulated by a set of non-polymerizable actin-like proteins. It remains to be established whether these proteins and actin are each encoded by separate genes.

PHYSARUM contains at least four distinct actin-binding proteins of approximately 42,000 molecular weight (MW)—actin, Cap42(b), Cap42(a) and fragmin¹⁻⁴. Only actin, which is present abundantly, forms filaments by self-assembly. The other three proteins do not form filaments but instead are able to block elongation of actin filaments by capping the fast-growing end of the filaments in a Ca^{2+} -dependent process²⁻⁵. Fragmin has an additional F-actin severing activity which leads to a rapid fragmentation of actin filaments when Ca^{2+} is present^{2,6-8}. Cap42(b) is phosphorylated by a specific kinase from *Physarum*; this phosphorylation changes its F-actin capping activity. Cap42(b) requires Ca^{2+} for its F-actin capping activity only when it is fully phosphorylated (when it is dephosphorylated, its F-actin capping activity is Ca^{2+} -independent)¹⁻⁴. The Cap42(b) kinase is highly specific for Cap42(b) and never phosphorylates either actin, fragmin or Cap42(a)¹. Hence, these four 42,000-MW proteins are functionally distinguishable from each other.

Like actin, Cap42(b) forms a very tight (1:1) complex with DNase I⁴. Consequently this complex is no longer phosphorylated by Cap42(b) kinase. Neither Cap42(a) nor fragmin binds to DNase I. Tight heterodimers form between actin and fragmin as well as between Cap42(b) and Cap42(a)¹⁻⁸. The actin-fragmin interaction is Ca^{2+} -dependent, whereas the Cap42(b)-Cap42(a) interaction is Ca^{2+} -independent. Their interactions appear to be highly specific, because a tight complex is never formed between actin and Cap42(a) or between Cap42(b) and fragmin. These findings indicate the possibility that actin is more closely related to Cap42(b), while fragmin shows a similar relationship to Cap42(a).

To clarify the structural relationships among these four actin-binding proteins, we have studied their immunochemical relations, peptide maps and specific binding to nucleotide triphosphates. The experimental data presented here strongly suggest that Cap42(b), Cap42(a) and fragmin are non-polymerizable but regulatory actin variants. In addition, we provide evidence that pp60^{sarc}, a tyrosine-specific kinase, preferentially phosphorylates fragmin in a Ca^{2+} -dependent manner.

Protein purity

Four distinct actin-binding proteins of 42,000 MW were separately purified from *Physarum* according to the procedures described previously^{1,4,7}. Because all four proteins have approximately the same molecular weight, we carefully tested for a possible cross-contamination in the preparations by various criteria. (1) The two DNase I-binding proteins, actin and Cap42(b), are much more negatively charged than fragmin or Cap42(a)¹, so that in a urea-polyacrylamide gel the former two proteins migrate much faster than the latter two. In our electrophoretic gel system, however, actin and Cap42(b) co-migrate with each other and are not separable unless either of them is phosphorylated selectively by specific kinases. This is also true for fragmin and Cap42(a). (2) A cyclic AMP-dependent protein kinase phosphorylates actin and never any of the three F-actin capping protein preparations⁴. (3) The Cap42(b) kinase from *Physarum* is highly specific for Cap42(b) and in no case phosphorylated any of the other three protein preparations¹. (4) As

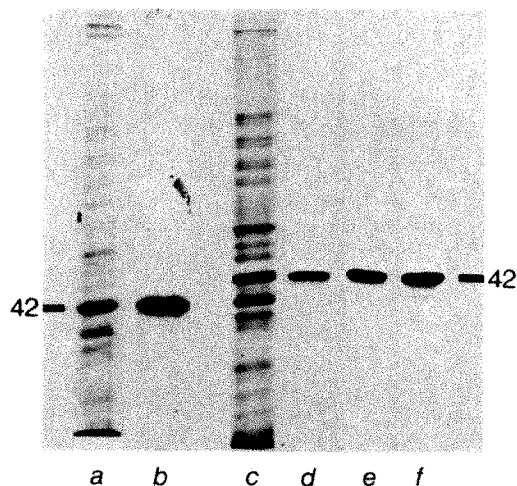


Fig. 1 Immunoblots of whole microplasmodial homogenates of *Physarum* with rabbit antibodies against actin, Cap42(b) and Cap42(a). Proteins in 1.5 μ l of packed microplasmodia of *Physarum* were separated by SDS-polyacrylamide (9% or 12%) slab gel electrophoresis, transferred onto nitrocellulose, incubated with four different IgG preparations from rabbits and then reacted with ¹²⁵I-protein A. a, c, Coomassie blue stained peptides of whole microplasmodial homogenates in SDS-polyacrylamide (9% or 12%) gels. b, d, e, f, Autoradiograms of the immunoblots: b, IgG against SDS-denatured Cap42(b); d, IgG against native Cap42(b); e, IgG against SDS-denatured Cap42(a); f, IgG against oxidized actin. The marker 42 indicates the molecular weight ($\times 10^{-3}$) of standard peptides.

we described in detail here, pp60^{sarc} preferentially phosphorylates fragmin but none of the other three protein preparations (Fig. 5). (5) Cap42(a) is only able to inhibit almost complete the phosphorylation of Cap42(b) in the presence of actin at Ca^{2+} (ref. 1). (6) The actin sample never showed any significant F-actin capping activity⁴. Using these criteria, which in each case are unique for only one of the four proteins, we could be sure that there was little cross-contamination in any of the four protein preparations used in our experiments.

Immunological cross-reactivity

To examine whether these four proteins share any common antigenic sites, we raised polyclonal antibodies in rabbits against Cap42(a) (SDS-denatured), Cap42(b) (native or SDS-denatured), fragmin (native) and actin oxidized by performic acid⁹ to enhance its immunogenicity. IgGs were purified by affinity chromatography on protein A.

Immunoblots show that IgGs against actin, Cap42(b) and Cap42(a) react almost exclusively with peptides of 42,000 MW in the whole microplasmodial homogenate (Fig. 1). Occasional anti-actin reacts with two additional peptides of 55,000 and 90,000 MW but much more weakly than with 42,000-MW peptides. The peptide of 55,000 MW might be α -tubulin because region of α -tubulin has been shown to contain an amino ac

Fig. 2 Immunological reactions of four distinct rabbit antibodies with the four separable proteins of 42,000 MW in *Physarum*. Rabbit IgGs against SDS-denatured Cap42(b) (a), native Cap42(b) (b), SDS-denatured Cap42(a) (c) and oxidized actin (d) were affinity-purified on protein A-Sepharose and their immunological reactions with native actin, Cap42(b), Cap42(a) and fragmin were determined by solid-phase radioimmunoassays (RIAs) including ^{125}I -labelled protein A as a radioactive indicator for IgG. Briefly, 1 μg of antigen in TDSA buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 15 % sucrose, 0.02 % NaN_3) was dried onto the surface of an RIA well. After washing with TTX buffer (10 mM Tris-HCl, pH 7.5, 0.1 % Triton X-100, 0.15 M NaCl, 0.1 % bovine serum albumin, 0.02 % NaN_3), the wells were incubated with various dilutions of IgG in the same buffer at 25°C for 1 h. After removal of unbound IgGs by washing with TTX buffer, 0.3 ng of ^{125}I -protein A (25,000 c.p.m. per well) in TTX buffer was added and incubated at 25°C for 1 h. Unbound protein A was then removed by washing with TTX buffer, and the radioactivity of protein A bound to IgG was measured. Although the data are not shown, the rabbit IgG against *Physarum* actin reacted equally well with oxidized and native actin from *Physarum* but not with rabbit skeletal muscle actin.

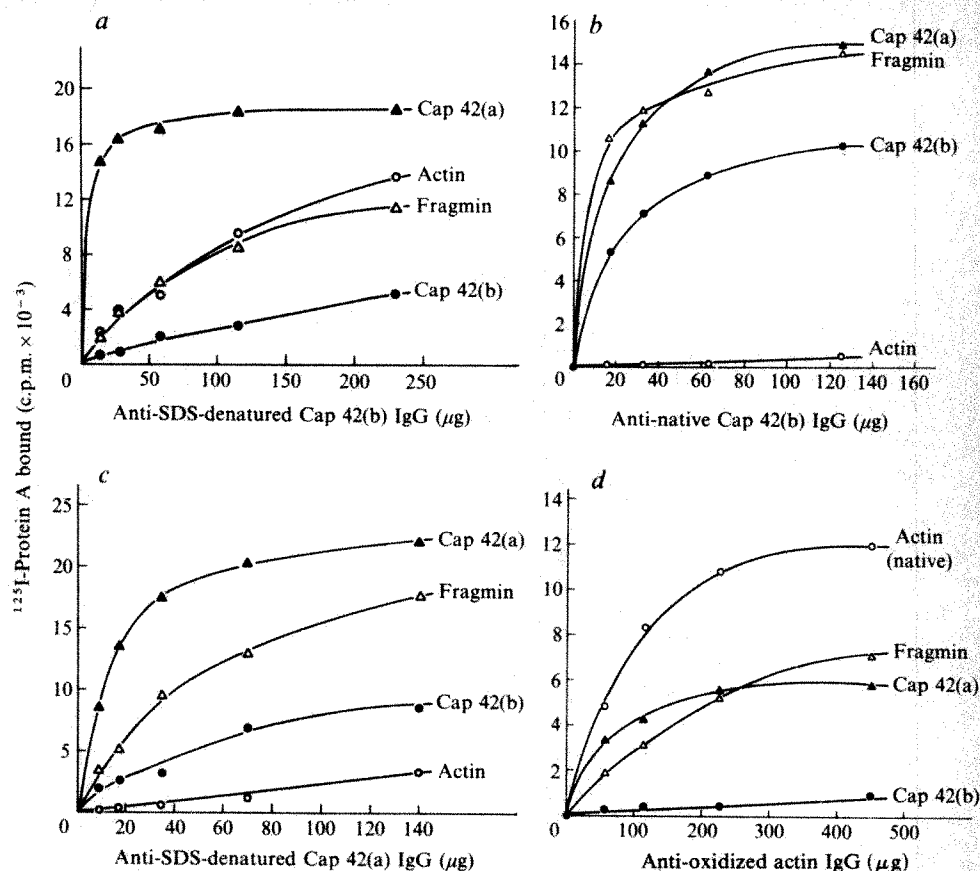


Table 1 Functional differences and sequence homology between four proteins

	Actin	Cap42(b)	Cap42(a)	Fragmin
Polymerization	+	-	-	-
Phosphorylation (Ser)	+	-	-	-
Phosphorylation (Thr)	-	+	-	-
Capping F-actin	-	+	+	+
Severing F-actin	-	-	-	+
Phosphorylation (Tyr)	-	-	-	+
Binding to DNase I	+	+	-	-
Binding to UTP	-	++	+	-
Binding to ATP	+	+	++	+
Binding to IgG I	++	+	+++	++
Binding to IgG II	-	+	++	++
Binding to IgG III	-	+	++	++
Binding to IgG IV	-	+	++	++
Binding to IgG V	++	-	+	+

Phosphorylation either by cyclic AMP-dependent protein kinase from bovine heart⁴ (Ser), or by *Physarum* Cap42(b) kinase¹ (Thr), or by pp60^{src} (Tyr). The abbreviations used for rabbit antibodies are: IgG I, anti-SDS-denatured Cap42(b); IgG II, anti-native Cap42(b); IgG III, anti-SDS-denatured Cap42(a); IgG IV, anti-fragmin; IgG V, anti-oxidized actin.

sequence very similar to that of actin¹⁰. Another as yet unidentified peptide of 90,000 MW obviously cross-reacts with this IgG at least in its denatured form. IgGs against fragmin, affinity purified on fragmin-Sepharose, were shown previously to react only with peptides of 42,000 MW in crude extracts of *Physarum*¹¹.

The cross-reactivity of these antibodies with the four distinct 42,000 MW actin-binding proteins was determined by solid-phase radioimmunoassays (RIAs). Rabbit antibodies against SDS-denatured Cap42(b) react not only with Cap 42(b) but also with actin, Cap42(a) and fragmin (Fig. 2a). Preincubation of this IgG fraction with *Physarum* actin reduces the binding to

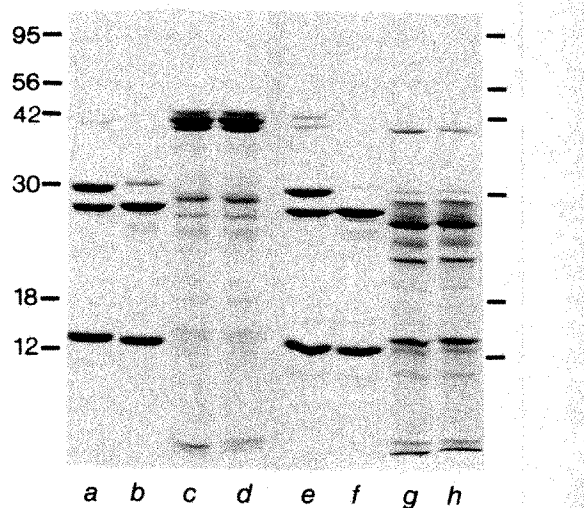


Fig. 3 Tryptic peptide maps of Cap42(b), actin, fragmin and Cap42(a). After boiling in the presence of 0.5 % SDS and 40 mM DTT and a 5× dilution into TDSA buffer, 20 μg of each protein were digested at 35°C for 10 min with 0.1 or 0.4 μg of trypsin, and their tryptic fragment patterns were analysed by SDS-polyacrylamide (16%) gel electrophoresis. a-d, Digested with 0.1 μg of trypsin; e-h, digested with 0.4 μg of trypsin. a, e, Cap42(b); b, f, actin; c, g, fragmin; d, h, Cap42(a). The markers indicate molecular weights ($\times 10^{-3}$) of standard peptides.

all four proteins (data not shown). On the other hand, rabbit antibodies elicited by immunization with native Cap42(b) (Fig. 2b) and SDS-denatured Cap42(a) (Fig. 2c) as well as native fragmin (data not shown) react more strongly with fragmin, Cap42(a) and Cap42(b) than with actin. Rabbit antibodies against oxidized actin from *Physarum* react with native actin from *Physarum* as well as with fragmin and Cap42(a), but not with Cap42(b) (Fig. 2d). These data indicate that actin, fragmin,

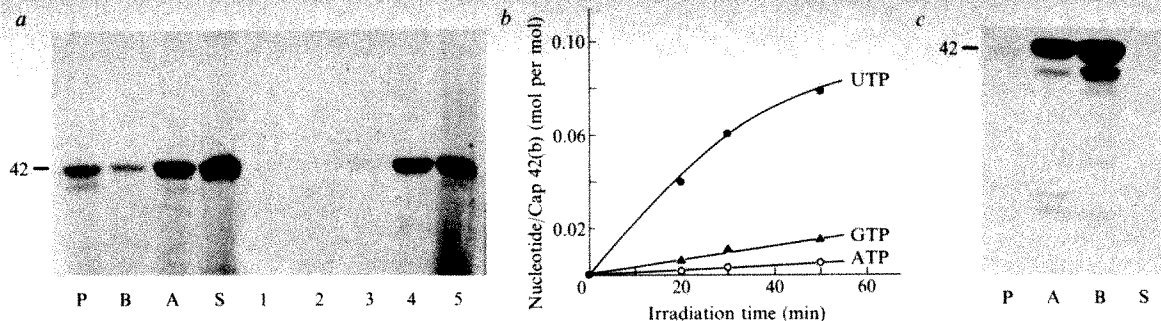


Fig. 4 *a*, Direct photoaffinity labelling by [α - ^{32} P]ATP of four proteins of 42,000 MW from *Physarum*. 2 μ M actin, Cap42(b), Cap42(a) or fragmin were preincubated with 80 μ M [α - ^{32} P]ATP (20 μ Ci) in 50 μ l of TDSA buffer at 0°C for 30 min, and then irradiated by UV light (λ 254 nm) at a distance of 2–3 cm for 30 min according to the procedure described previously²². The radioactive ATP covalently bound to each peptide was separated from free ATP by SDS-polyacrylamide gel (9%) electrophoresis and visualized by subsequent radioautography. Lane P, actin; lane B, Cap42(b); lane A, Cap42(a); lane S, fragmin. Effects of a large excess (10 mM) of non-radioactive ATP and other nucleotide triphosphates as well as diphosphate and monophosphate on the direct photoaffinity labelling of Cap42(a) by [α - ^{32} P]ATP were also examined under the same conditions and compared with the control *a*: lane 1, ATP; lane 2, GTP; lane 3, UTP; lane 4, pyrophosphate; lane 5, phosphate. Only nucleotide triphosphates almost completely inhibited the covalent binding of radioactive ATP to Cap42(a). *b*, Covalent binding of UTP, GTP and ATP to Cap42(b) by UV irradiation. The time course of the direct photoaffinity labelling of Cap42(b) was followed in the presence of 80 μ M [α - ^{32} P]UTP, [α - ^{32} P]GTP or [α - ^{32} P]ATP at 0°C for the indicated time by filter paper assay as described previously²². *c*, Only Cap42(b) and Cap42(a) covalently bound UTP by UV irradiation. The direct photoaffinity labelling of Cap42(b) by [α - ^{32} P]UTP was also compared with those of the other three proteins of 42,000 MW in the same conditions as described for *a*. Lane P, actin; lane A, Cap42(a); lane B, Cap42(b); lane S, fragmin.

Cap42(a) and Cap42(b) share some antigenic sites, that the three F-actin capping proteins have a subset of antigenic sites in common that are not shared with actin and that Cap42(b) lacks some antigenic sites which are commonly present in actin, Cap42(a) and fragmin. Among the three F-actin capping proteins, Cap42(a) and fragmin are very similar to each other in these immunochemical reactions.

Tryptic peptide maps

One-dimensional tryptic peptide maps of Cap42(b) are very similar but not identical to those of actin (Fig. 3), whereas maps of Cap42(a) are almost identical to those of fragmin. The maps of these two pairs of similar proteins differ considerably from each other, suggesting that the four proteins may fall into two groups. One pair is Cap42(a) and fragmin, neither of which binds to DNase I. The other pair, Cap42(b) and actin, both bind to DNase I.

Photoaffinity labelling

Actin has at least one binding site for ATP or ADP¹² and can be labelled with [α - ^{32} P]ATP by a photoaffinity technique (Fig. 4*a*). The same treatment covalently labels Cap42(b), Cap42(a) and fragmin (Fig. 4*a*), with Cap42(a) being the most heavily labelled. The binding of radioactive ATP to Cap42(a) is inhibited almost completely by a large excess (~100 times) of non-radioactive UTP, GTP or ATP, indicating that both UTP and GTP probably share the same binding site on Cap42(a) with ATP. Cap42(b) is most heavily labelled by [α - ^{32} P]UTP among the three nucleotide triphosphates tested (Fig. 4*b*). Furthermore, the binding of radioactive UTP to Cap42(b) is inhibited almost completely by a large excess of non-radioactive ATP, GTP or UTP (data not shown), again indicating that these three nucleotide triphosphates probably bind to the same site on Cap42(b). Interestingly, as shown in Fig. 4*c*, neither actin nor fragmin binds UTP, although both proteins bind ATP (see also Fig. 4*a*).

Phosphorylation of fragmin by pp60^{sarc}

pp60^{sarc} is a phosphopeptide of 60,000 MW encoded by the *sarc* gene which is solely responsible for the Rous sarcoma virus-induced transformation of avian cells^{13,14}. This protein is associated with a protein kinase activity which phosphorylates preferentially tyrosine residues of several proteins including the heavy chain of IgG, vinculin, casein and pp60^{sarc} itself^{14–17}. Recently a peptide of 42,000 MW called pp42 was shown to be phosphorylated *in vivo* at its tyrosine residues during Rous sarcoma virus-induced transformation¹⁸. pp42 appears to be distinct from any of the polymerizable actin variants, however, as its isoelectric

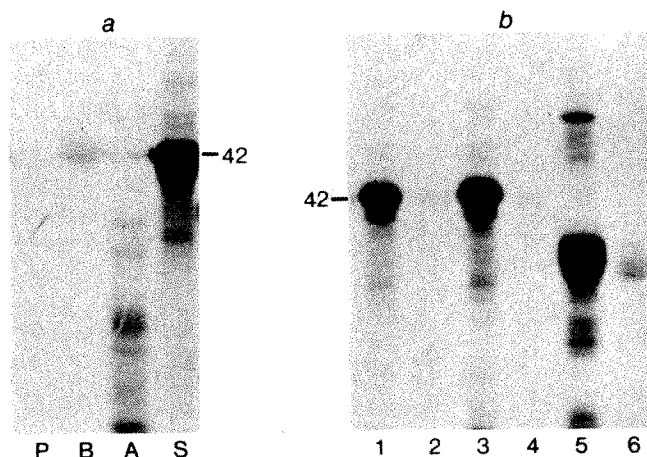


Fig. 5 *a*, Only fragmin was phosphorylated by pp60^{sarc}. 20 μ g of the four proteins of 42,000 MW were separately incubated with pp60^{sarc} at 35°C for 30 min in the presence of 40 μ M [γ - ^{32} P]ATP, 10 mM Mg²⁺ and 0.2 mM Ca²⁺. The phosphorylation of each peptide was analysed by SDS-polyacrylamide (9%) gel electrophoresis and subsequent autoradiography. Lane P, actin; lane B, Cap42(b); lane A, Cap42(a); lane S, fragmin. The marker (42) indicates peptides of 42,000 MW. *b*, Ca²⁺ stimulated the phosphorylation of fragmin by pp60^{sarc}. The phosphorylation of fragmin by pp60^{sarc} was carried out in the presence or absence of Ca²⁺, otherwise in the same conditions as described for *a*. Lane 1, fragmin + pp60^{sarc} + 1 mM EGTA; lane 2, fragmin + 1 mM EGTA; lane 3, fragmin + pp60^{sarc} + 0.2 mM Ca²⁺; lane 4, fragmin + 0.2 mM Ca²⁺; lane 5, α -casein + pp60^{sarc} + 0.2 mM Ca²⁺; lane 6, α -casein + 0.2 mM Ca²⁺. Marker 42 indicates fragmin.

point is around 7.0 instead of 5.4 (= *pI* of actin) and its biological activity and the effect of phosphorylation are still unknown. Interestingly, microinjection of Cap42(a+b), an equimolar complex of Cap42(a) and Cap42(b), causes a rapid and reversible disruption of actin stress fibres in mammalian fibroblasts¹⁵ which is similar to that occurring at an early stage of virus-induced transformation of the cells. As pp42 might be a non-polymerizable actin variant, we have asked if pp60^{sarc} is able to phosphorylate some of the three F-actin capping proteins of 42,000 MW from *Physarum*. A preparation of pp60^{sarc} (gift of Drs Y. Sugimoto and R. L. Erikson) was purified to homogeneity from European field vole cells transformed by the Schmidt-Ruppin strain of Rous sarcoma virus (unpublished data). We found that pp60^{sarc} preferentially phosphorylates fragmin in a Ca²⁺-dependent manner (Fig. 5). Neither actin nor Cap42(b)

nor Cap42(a) serve as a substrate for pp60^{src}. These results provide additional evidence for our hypothesis that fragmin is functionally distinct not only from actin and Cap42(b) but also from Cap42(a). It remains to be determined, however, whether F-actin capping and severing activities of fragmin are significantly affected by its tyrosine phosphorylation.

Conclusions

Physarum has at least four actin-binding proteins of 42,000 MW: actin, Cap42(b), Cap42(a) and fragmin, that can be distinguished functionally (Table 1), but the cross-reactivity of antibodies, peptide maps and photoaffinity labelling by nucleotide triphosphates provide evidence that the four proteins have common as well as unique structural features (Table 1). We suggest that Cap42(b), Cap42(a) and fragmin are variants of actin that can bind to the fast-growing end of actin filaments but cap the polymer because they cannot propagate further growth. The four proteins fall into two closely related pairs: actin and Cap42(b), and Cap42(a) and fragmin, actin and fragmin share; the fewest common features. Based on all the available data concerning the structure and function of the four proteins, we suggest that they are related to each other in the following order—actin, Cap42(b), Cap42(a), fragmin. Proof of these relationships will be provided by the primary structure of the proteins and their genes. Preliminary amino acid sequence data

(ref. 20 and J. Vandekerckhove *et al.*, in preparation) show that even fragmin has regions identical to actin, although the former has a unique sequence region not shared by the latter. Thus, it seems clear that at least two of these proteins, actin and fragmin, are derived from two distinct actin genes. Similarly, the amino acid sequences of Cap42(b) and Cap42(a) will establish whether they are simply post-translationally modified forms of actin or fragmin, or, as we believe, whether all four proteins are derived from different, distinct genes. Genetic analysis of *Physarum* actin genes suggests that *Physarum* contains at least four and probably five different actin genes²¹. We speculate that these genes code for actin, Cap42(b), Cap42(a) and fragmin and that they were all derived from a common ancestral gene by its duplication and subsequent mutation or rearrangement during evolution of *Physarum*. Finally, because all four distinct 42,000 MW actin-binding proteins in *Physarum* are probably actin variants with different biological activities, we propose to call actin and fragmin by more logical and uniform terms implying both their unique functions and molecular weights. We have called the major polymerizable actin variant Pol 42, and fragmin, an F-actin severing variant, Sev 42.

We thank Dr Thomas D. Pollard at Johns Hopkins University for his critical reading of the manuscript and many valuable suggestions and Dr Wolfgang Baltes and Ms Rita Ohnheiser for their technical assistance. G.I. is recipient of grant Is 25/3-1 from the Deutsche Forschungsgemeinschaft.

Received 5 April; accepted 28 August 1984.

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LETTERS TO NATURE

Improved limits on small-scale anisotropy in cosmic microwave background

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As a remnant of the early Universe, the cosmic microwave background provides unique information on the initial conditions from which matter has evolved to form the structures we see today. All efforts to detect small-scale structure in this radiation have so far been unsuccessful (ref. 1 and refs therein)^{1–4}. Nevertheless, upper limits set on possible underlying fluctuations restrict the range of physical models for perturbations of the density in the early Universe. Our search for small-scale anisotropy in the background radiation has now resulted in a lowering of the upper limit on root-mean-square fluctuations ($\Delta T_{\text{r.m.s.}}$) observed at an angular scale of ~ 4 arc min to $\Delta T_{\text{r.m.s.}}/T < 2.1 \times 10^{-5}$ at the 95% confidence level (where $T = 2.7$ K, the temperature of the background radiation). The actual limits deduced from our experiment depend on the model assumed for the unseen fluctuations. Several possibilities are discussed as well as the implications this new measurement has for various cosmological models.

The 140-foot telescope of the US National Radio Astronomy Observatory (NRAO), with the K-band maser receiver mounted at the Cassegrain focus, is an excellent instrument for high-

sensitivity continuum observations². This system routinely achieves a temperature of 50 K with 370 MHz bandwidth, centred at 19.5 GHz. Moreover, it is exceedingly stable for long integrations and consistently reaches a sensitivity of $\Delta T_{\text{r.m.s.}} = 10^{-4}$ K (antenna temperature) for 2 h of observation in the best weather conditions (clear sky and ambient temperature below 0°C). At this frequency, the half-power beamwidth is about 1.5 arc min.

We have used this system to search for small-scale anisotropy in the microwave background radiation. Although our first observations were made in December 1980, we shall be concerned here only with data collected since December 1982, which are of much better quality than previous data. Good data were obtained from 27–31 December 1982 (82 h) and 16–20 May 1983 (night-time only, 30 h): these have been discussed in ref. 2. The new observations reported here were made between 25 February and 5 March 1984 (88 h) with additional data taken between 3 and 7 January 1984 (4 h) in a filler programme scheduled with other observations. Thus, a total of 174 h of high quality data were obtained from these observing runs out of a total of 445 h scheduled initially.

Fluctuations in the gain of the receiver, as well as variable contributions of radiation from the atmosphere and the ground, are important sources of systematic errors in this kind of measurement. To overcome these problems, the telescope beam is switched at a rate of 3.33 Hz between two positions in the sky, separated by 4.5 arc min, by mechanically oscillating the secondary reflector. The receiver output, δT , which is detected synchronously with beam position, is thus a measure of the difference between the antenna temperatures of the radiation

collected from the two positions in the sky with the addition of a variable offset, due to the differences of atmospheric and ground radiation in the two beams. To eliminate this variable offset, the telescope is periodically moved by 4.5 arc min to place a given sky position (called the field) alternately in the two beams. This gives two sets of data for each field

$$\delta T_{\text{on}} = T_{\text{Field}} - T_{\text{ref2}} + T_{\text{offset-on}}$$

and

$$\delta T_{\text{off}} = T_{\text{ref1}} - T_{\text{Field}} + T_{\text{offset-off}}$$

where T_{ref1} and T_{ref2} correspond respectively to the westerly and easterly reference positions, 4.5 arc min away from the corresponding field. The two offset signals in equation (1) cannot be measured with sufficient accuracy to subtract them; furthermore, they are slowly varying functions of time and telescope position. By accurately timing the off-on observing sequence, one hopes that both offsets will be equal and will cancel in the expression

$$\begin{aligned} \Delta T_{\text{Field}} &= (\delta T_{\text{on}} - \delta T_{\text{off}})/2 \\ &= T_{\text{Field}} - (T_{\text{ref1}} + T_{\text{ref2}})/2 \end{aligned} \quad (2)$$

which is the quantity measured for each field. The atmospheric and ground effects are too complex to predict at the 10^{-4} K level, so one must try and see what happens.

The 12 candidate fields were chosen as close to the north celestial pole as safe operation of the 140-foot telescope allows: declination (1950.0) $86^{\circ}51'$ with right ascensions: 1 h 30 min, 3 h 30 min, ..., 23 h 30 min. This allows 2 h of observation of each field on each day, while minimizing the motion of the telescope with respect to the ground and the atmosphere. At this declination and with a 4.5 arc min beam separation, the telescope goes through the same range of positions with respect to the ground for an off-on pair if the on-scan is started 5 min 3 s after the off-scan. As changing the position of the telescope takes about 15 s, the best cancellation of T_{offset} is achieved with an integration time of 4 min 48 s for each scan. We emphasize that keeping the residual systematic errors below 10^{-4} K requires the atmospheric and ground contributions to be repeatable to better than one part in 10^5 over times longer than the 10 min duration of the off-on sequence. It is remarkable that, with good weather conditions, this requirement is indeed satisfied with this particular telescope.

After correcting the data for the beam efficiency of the antenna (55%) and converting to units of thermodynamic temperature, we compute the mean values $\Delta T_{\text{Field}}^i$ for the observations of each field on each day (i). The associated standard deviations, σ_{Field}^i , are calculated from the scatter among the different off-on measurements (typically 12 on each day), and are an estimate of the statistical uncertainty in the mean, after 2 h of observation. A typical value for data taken with clear sky is $\sigma_{\text{Field}}^i = 0.25 \times 10^{-3}$ K, whereas one expects 0.14×10^{-3} K from system noise alone. The excess noise at this stage is due to a $1/f$ component in the noise power spectrum of the receiver and to the variable components of the atmospheric and ground contributions to δT_{on} and δT_{off} .

The weighted average of the values of $\Delta T_{\text{Field}}^i$ from the different days in which each field was observed yield the final values $\bar{\Delta T}_{\text{Field}}$ plotted in Fig. 1. The errors associated with each point correspond to the standard deviations, σ_{Field} , estimated from the measurement statistics in the usual way. It is crucial to estimate accurately the errors in Fig. 1 because the data will be used to decide whether a significant sky signal has been detected or, alternatively, at what level a sky signal is excluded. A major concern is whether $\Delta T_{\text{Field}}^i$ changes from day to day owing to slow changes in $T_{\text{offset-on}} - T_{\text{offset-off}}$. This can be checked by comparing the measured day-to-day scatter in $\Delta T_{\text{Field}}^i$ with the corresponding values of σ_{Field}^i . Figure 2 shows a histogram of the weighted residuals $(\Delta T_{\text{Field}}^i - \bar{\Delta T}_{\text{Field}})/\sigma_{\text{Field}}^i$ for the 87 2-h observations. The histogram matches the gaussian distribution expected if all the day-to-day scatter were due to fluctuations

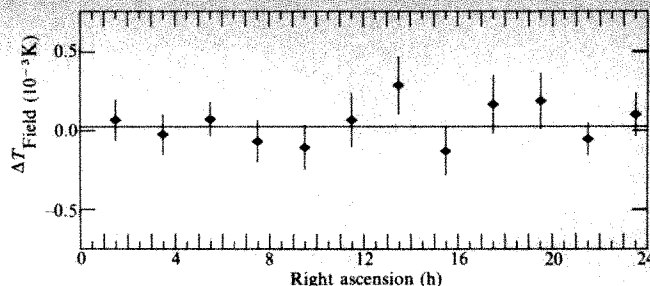


Fig. 1 Final averages for the observed fields. The solid line indicates the overall weighted average, which is $\Delta T_{\text{ave}} = (26 \pm 39) \times 10^{-6}$ K. ΔT_{Field} is the difference in thermodynamic temperature between the field and the average of two reference positions, 4.5 arc min away on opposite sides. (See equation (2).)

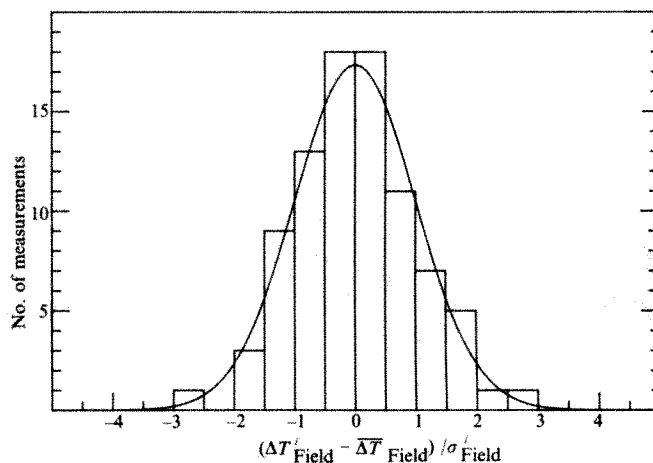


Fig. 2 The day-to-day scatter in measurements averaged over ~ 2 h. As the residuals from the field averages $\Delta T_{\text{Field}}^i$ are used, the possible sky signals have been removed. If there were no extraneous day-to-day effects, the histogram would fit the gaussian curve shown; it does so reasonably well.

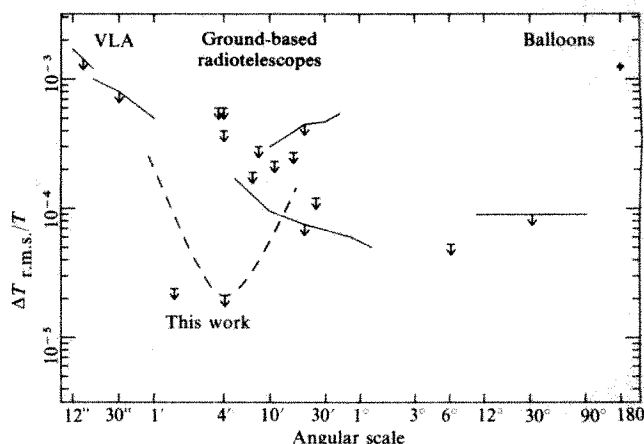


Fig. 3 ∇ , Upper limits (95% confidence level) on anisotropy of the 2.7 K radiation at various angular scales. The Very Large Array (VLA) results^{3,4} are new and will probably be improved. Some of the points between $1'$ and 1° have been revised¹ from the values given in the original references. Anisotropy at large angular scales⁸ must be measured from balloons or satellites because of atmospheric noise.

contained in σ_{Field}^2 . Indeed, the corresponding χ^2 is 78 with 75 degrees of freedom. Therefore the values of σ_{Field} displayed in Fig. 1 are not contaminated by day-to-day effects and are accurate estimates of the measurement errors.

The weighted average of the values in Fig. 1 is $\Delta T_{\text{ave}} = (26 \pm 39) \times 10^{-6}$ K. As none of the 12 points differs significantly from this value, no anisotropy has been detected at a level of $\sim 2\bar{\sigma}_{\text{Field}}$. A more stringent limit can be set by using all the data in a statistical argument, although this requires adopting a model for the anisotropy. The underlying sky signal is expected to be gaussian, distributed between the 12 field positions (separation $> 1.6^\circ$), and can be characterized by its standard deviation, σ_{sky} . Assuming there is no correlation between the signals arriving from a given field and associated reference positions (4.5 arc min scale), the r.m.s. residual in ΔT_{Field} due to a true sky signal is (from equation 2)

$$\sigma_{\text{r.m.s.}}^2 = (4\sigma_{\text{sky}}^2 + \sigma_{\text{sky}}^2 + \sigma_{\text{sky}}^2)/4 = 1.5\sigma_{\text{sky}}^2 \quad (3)$$

In this case, the off-on procedure is a probe of σ_{sky} with $\sqrt{1.5}$ efficiency. This efficiency factor depends on the angular autocorrelation that is assumed for the possible sky signal and must be evaluated separately for each different model.

The Neyman-Pearson lemma prescribes the optimal statistic for testing a hypothetical value of σ_{sky} . In our case the best statistic is essentially a weighted χ^2 (ref. 2), which yields

$$\sigma_{\text{r.m.s.}} = (0.1 \pm 4.7) \times 10^{-5} \text{ K} \quad (4)$$

and an upper limit

$$\sigma_{\text{r.m.s.}} < 7.8 \times 10^{-5} \text{ K (95\% confidence level)} \quad (5)$$

Using equation (3) and $T = 2.7$ K gives

$$\sigma_{\text{sky}}/T < 2.4 \times 10^{-5} \text{ (95\% confidence level)} \quad (6)$$

Limits on anisotropy from different experiments are difficult to compare. Because various scanning patterns and statistical methods have been used, careful statements of limits on a particular model should examine each measurement separately. Figure 3 shows the present experimental limits, most of which assume that the underlying anisotropy is uncorrelated gaussian noise. For this assumption the result of equation (6) applies at an angular scale of 1.5 arc min.

A more general way to interpret our result is to take the Fourier transform of the scan pattern (three gaussian beams 1.5 arc min across, separated by 4.5 arc min) and then to average it over the orientation angle. Thus, the limit shown in Fig. 3 (dashed line) assumes that all of the anisotropy is the result of gaussian sinusoidal fluctuations of a certain scale. The strongest limit corresponds to a scale (half wavelength) of 4 arc min and is

$$\Delta T_{\text{r.m.s.}}/T < 2.1 \times 10^{-5} \text{ (95\% confidence level)} \quad (7)$$

which is more stringent than the one given in equation (6) because the sinusoidal fluctuations are anticorrelated on the scale of our beam-throw, increasing the efficiency of the observing procedure for this kind of fluctuation.

A physical interpretation of the results requires a model for the nature of the underlying fluctuations. Most authors assume fluctuations with a spectrum of the form $(\delta\rho/\rho) \propto k^n$, for the dominant contribution to the mass of the Universe at the time of decoupling of matter and radiation (a redshift of $\sim 1,000$). The amplitudes of the fluctuations are fixed by matching the evolved autocorrelation function of the matter to the data available from studies of galaxy clustering. The details of the interaction between the mass components and the background radiation determine the anisotropy required by each particular model. Comparing our results with recent calculations⁵ rules out those models in which the initial fluctuations are adiabatic and dominated by baryonic matter. Baryon-dominated universes in which the initial perturbations are isothermal are currently out of fashion but are not seriously constrained by our limit: for $n < 0$ one needs to satisfy $0.1 < \Omega < 0.3$ (Ω is the ratio of the density of the Universe to that of an Einstein-de Sitter universe),

whereas no restriction is imposed if $n \geq 0$. Models dominated by massive neutrinos require $\Omega \sim 1$, although they suffer other problems^{6,7}. More exotic possibilities are models in which the dominant component is in the form of yet undiscovered cold, dark matter, such as axions or photinos. These models require that $\Omega H > 0.2$ (H is Hubble's constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$).

Finally, we must caution that the analysis of our data rests on the assumption that the underlying fluctuations have a gaussian distribution: if untrue, the theoretical predictions could be uncertain by factors of 2. Although there is a conflict between the theoretical models and the experimental data, the fate of the various models is still far from settled.

We thank Drs C. Brockway and R. Fisher for their continuous improvement of the telescope, receiver and data acquisition system, and all the NRAO-Green Bank staff for their help and constant vigilance. This research was supported in part by the NSF. The National Radio Astronomy Observatory of the USA is operated by Associated Universities, Inc. under contract with the NSF.

Received 20 August; accepted 18 September 1984.

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Effects of pulsation and mass loss on stellar evolution

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There is much evidence that many post-main sequence stars are losing mass, that the integrated mass loss is sufficient to affect their evolution and that epochs of substantial mass loss are normal for almost all stars. However, neither the precise timing, nor the mechanisms, nor the full consequences of this mass loss are yet well understood. Stellar pulsation may play a key role: it is closely associated with evolutionary phases where substantial mass loss occurs, and there are good physical reasons to expect pulsation to cause, or at least greatly to enhance, mass loss. Here we point out that there are many possible consequences, some not previously recognized, of pulsation-related mass loss for the evolutionary behaviour of post-main sequence stars. We advance the hypothesis that most mass loss of evolutionary significance is closely related to stellar pulsation.

We have been modelling the atmospheric dynamics of Mira variables, which exhibit long-period, large-amplitude radial pulsation and are known to have high mass-loss rates. Recently, we have begun using a similar approach to model other pulsating giants. There are several ways in which pulsation can lead to mass loss, some relevant to Mira variables and some to other classes of stars. Indeed, it now appears almost inevitable that pulsation not only can but does lead to significant mass loss in almost every case, through transfer of momentum and energy to the outer atmosphere. Steady pulsation of even moderately large amplitude generates shock waves and causes great extension of the stellar atmosphere, thereby strongly enhancing all mass-loss processes. Radiative cooling, which depends on collisions, becomes so slow in the low-density outer atmosphere (now a very large region with considerable total mass) that dynamic processes there become effectively adiabatic¹. Non-radiative energy transfer to the outer atmosphere by pulsation-induced shocks can thus have an important effect. In RR Lyrae

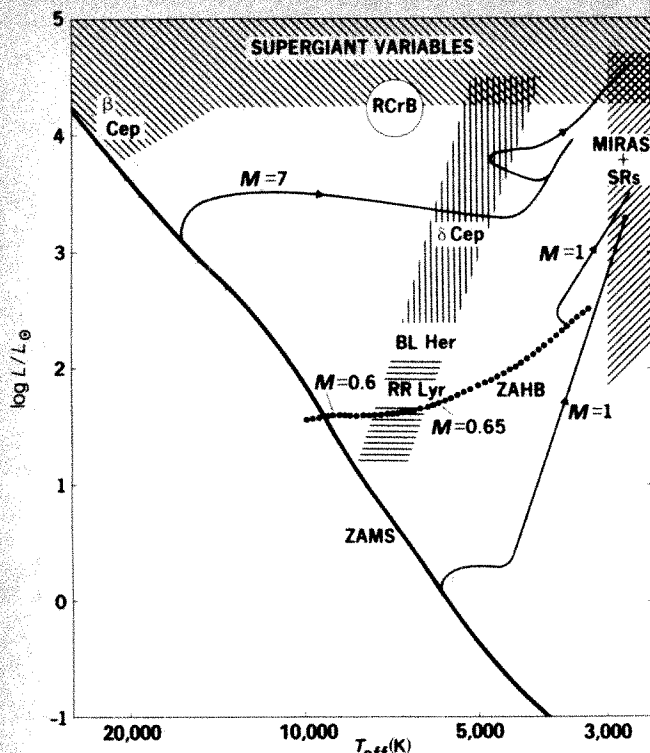


Fig. 1 Hertzsprung-Russell diagram (luminosity versus temperature) showing the most important regions of pulsational instability. Standard zero-mass loss evolutionary tracks of mass equal to $1 M_{\odot}$ and $7 M_{\odot}$ stars of solar composition are sketched in for reference, as well as the zero-age horizontal branch for core He burning stars^{4,5,9}.

models, for example, it heats the outer atmosphere sufficiently to produce a hot corona and cause a substantial coronal wind. In contrast, the outer atmospheres of Mira models remain cool; dust grains form there and the density in the condensation region is increased by many orders of magnitude compared with static models; dust effects dominate and radiation pressure on the grains drives strong winds similar to those observed. With modest changes in stellar parameters, this Mira-like behaviour grades smoothly into models with optically thick circumstellar dust shells and still more massive winds, simulating the infrared OH-IR sources.

Prodigious mass loss must occur before or during the ascent of the asymptotic giant branch, because white dwarf stars, with masses typically of $0.6\text{--}1.0 M_{\odot}$, are formed from progenitors with masses up to 5 or $6 M_{\odot}$, or more^{2,3}. Attempts to reproduce observational evidence, including not only direct comparisons of calculated and observed properties of asymptotic giant branch stars but also the effects of nucleosynthesis and mass recycling on galactic chemical evolution, indicate that the mass-loss process must be discontinuous, with a terminal 'superwind' stage removing at least the last few tenths of a solar mass of envelope material in a time which is short on the nuclear evolutionary time scale of these stars². This has a natural explanation, supported by observed mass-loss rates, in terms of rapid pulsation-induced mass loss during the brief but very common Mira stage of evolution at the tip of the asymptotic giant branch⁴.

Calculations indicate that the evolutionary track of helium core-burning (horizontal branch) stars with $M \sim M_{\odot}$ will lie to the red of the RR Lyrae instability strip; only stars with low metallicity and/or masses $\leq 0.7 M_{\odot}$ evolve far enough into the blue during this stage to produce RR Lyrae stars^{5,6}. Most of this mass is presumably lost during the first ascent of the star up the red giant branch. Near the end of this ascent, these stars probably enter the same pulsational instability region which the Mira and semiregular variables occupy at higher luminosity. A reasonable hypothesis is that pulsation near the tip of the red

giant branch provides the mechanism for mass loss needed to produce RR Lyrae stars.

Mass loss on the horizontal branch may also be indicated. Comparison of evolutionary calculations for horizontal branch stars with the observed blue-red horizontal-branch populations, and with the statistics of RR Lyrae stars, further indicates that within a given cluster there must be a modest range of masses along the horizontal branch: $\sim \pm 0.02 M_{\odot}$ (refs 5, 6). This has been assumed to be owing to stochastic variations in the mass loss on the red giant branch, but it can equally well be accounted for by assuming pulsation-induced mass loss during the RR Lyrae stage. As horizontal branch evolution is relatively slow, the mass-loss rates required are undetectably low at present: $\sim 10^{-10} M_{\odot} \text{ yr}^{-1}$ is sufficient to produce the range in masses needed to populate the blue and red horizontal branches of clusters.

This rate of mass loss from RR Lyrae stars has further implications for the morphology of cluster Hertzsprung-Russell (H-R) diagrams and for the ultimate fate of low-mass stars. One possible consequence of mass loss due to pulsation in the RR Lyrae stars is the trapping of these stars along the edges of the instability strips for fundamental and overtone pulsation. For those RR Lyrae stars evolving to the right in the H-R diagram, the effect of a decrease in mass is to force them to the left—possibly cancelling the evolutionary effect. If such a star were to tend to evolve into a region of the H-R diagram where, owing to its pulsation properties, its mass-loss rate would be large, it may become trapped by these competing effects at the blue boundary of the region. If the mass loss during overtone pulsation (which takes place at the blue side of the instability strip) is too low to inhibit evolution to the red, but the fundamental mode mass-loss rate is larger, the star may be trapped where the mode change occurs. This trapping would have an effect on the period distribution of RRc and R Rab stars similar to the 'hysteresis mechanism' proposed by von Albada and Baker⁷ and further elucidated by Stellingwerf⁸. In addition, trapping in the mode-switching region could explain the overabundance of double-mode RR Lyrae stars in clusters such as M15 (ref. 9). This trapping effect has as a further consequence, that quite low rates of mass loss are given more time to operate, thus producing greater net mass loss than would otherwise be supposed.

Mass loss during the horizontal branch pulsation stages should have observable effects on the RR Lyrae period distribution, on the luminosity difference between RR Lyrae and BL Her stars in the same cluster and on the relative population of the red and blue horizontal branches. Pulsation masses for the post-RR Lyrae BL Her stars are less than those for RR Lyrae stars¹⁰, as would be expected from our picture. The alteration of horizontal branch (HB) evolution by RR Lyrae pulsation-induced mass loss suggests the possible existence of a post-HB stage of pre-white dwarfs, which could explain the great blueward 'tail' of some cluster HBs. This would also lead to the formation of a separate group of low-mass ($\sim 0.5 M_{\odot}$) white dwarfs of high He content, from those stars whose He burning stage was terminated prematurely by RR Lyrae stage, pulsation-driven mass loss. Such post-HB stars may have been observed in the He shell-burning hot O subdwarfs, studied by Schonberner and Drilling¹¹.

Another form of trapping may be operating in the Cepheids, and may account for part of the persistent 'mass anomaly' between evolutionary and pulsationally derived (pulsation, beat and bump) masses^{12,13}. For shorter period Cepheids, models calculated on the assumption that mass loss on the red giant branch has removed enough mass before the Cepheid stage to reduce the masses to the 'beat' and 'bump' masses do not loop to the blue far enough to enter the instability strip. However, if the mass loss starts only when the star is already in the strip, this difficulty is removed. Furthermore, the stars spend most of their time near the blue end of the blue loops¹³. Mass loss during Cepheid pulsation may therefore have the effect of bringing this long-lived stage into the instability strip, allowing more time for the stars to lose mass. Observed mass-loss rates for at least some

Cepheids are in the range required if trapping operates: infrared excesses imply mass-loss rates of $\sim 10^{-6} M_{\odot} \text{ yr}^{-1}$ for SV Vul (period 45 days) and RS Pup (42 days); RS Pup appears to be surrounded by five shells of ejected material implying considerably higher mass-loss rates in the recent past^{14,15}. For the shorter period Cepheids, where the mass discrepancy is most serious, circumstellar lines indicative of material moving out with velocity $v > v_{\text{esc}}$ have been seen in the ultraviolet¹⁶.

Although various authors have argued that the Cepheid mass discrepancy lies entirely with the pulsation models, this conclusion depends on adopting a particular distance scale, for example, one with distance modulus ($m - M \geq 18.7$) for the Large Magellanic Cloud. If, as recent studies suggest¹⁷, the LMC distance modulus is nearer 18.5, the discrepancy between evolutionary and pulsation masses returns. Thus, for example, Schmidt¹⁸ finds only the evolutionary mass to be discrepant on his preferred distance scale.

There are many other known links between pulsation and mass loss. For example, we find pulsations reported for the heavily mass-losing R CrB class stars¹⁹; pulsations invoked to account for the large mass-loss rates of the Wolf-Rayet stars²⁰; infrared excesses detected for the pulsating RV Tauri stars¹⁴;

and variability and mass loss observed for the luminous supergiants, with a correlation between the amplitudes of variation and the mass-loss rates²¹.

The hypothesis that most, if not all, evolutionarily significant mass loss occurs as the direct or indirect result of stellar pulsation promises to resolve some long-standing problems in stellar evolution theory. It is a hypothesis which may be relatively easy to incorporate into future theoretical calculations, as the locations of regions of pulsational instability in the H-R diagram are well known. As in this hypothesis mass loss occurs quite rapidly during short episodes for most stars, evolutionary changes in the stellar parameters during such episodes will be small, allowing at least a partial separation of the mass-loss and evolutionary calculations. Efforts to observe and measure mass loss from pulsating variables as a function of their pulsation properties should provide further constraints on the mass-loss process.

We are continuing our investigations into the mechanisms and rates of mass loss caused by pulsation, for different types of stars using detailed numerical hydrodynamics. Here we have attempted only to give a brief and qualitative description of some of the major effects of pulsation in stellar systems. A full report on the methods and results will appear elsewhere.

Received 27 June; accepted 24 September 1984.

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Observations and theory of the solar semidiurnal tide in the mesosphere of Venus

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The Orbiter Infrared Radiometer experiment, aboard the 1978 Pioneer Venus mission, produced strong evidence for the existence of a semidiurnal solar tide in the mesosphere of Venus¹. At the same time, measurements *in situ* of radiative fluxes and cloud particle distribution, in other Pioneer Venus experiments, have provided much information about the structure of the solar heating and infrared cooling there²⁻⁴, making it possible to calculate the thermal tidal forcing functions with confidence, and also allowing good estimates of the radiative dissipation rates to be made. Because the structure of tidal fields depends sensitively on that of the background zonal mean wind velocity, $U(\theta, z)$, a knowledge of the sources and sinks of tidal energy and of the actual structure of the tide should allow one to infer the behaviour of U . We show here that a physically reasonable mean flow can be found that leads to theoretically predicted tides that are in excellent agreement with those observed. Our zonal mean flow shows a maximum velocity of $\sim 130 \text{ ms}^{-1}$ at 70 km, decreasing dramatically to ~ 0 at 88 km, and a jet-like meridional structure that deviates strongly from solid-body rotation.

The observed semidiurnal thermal tide is taken to be the solar-fixed, wavenumber-two component of the temperature field derived from the Orbiter Infrared Radiometer observations. This instrument made measurements in five different channels,

with vertical response or weighting functions peaked at about 99, 86, 76, 68 and 64 km for channels 1 through 5 respectively. We are particularly interested in the region 70-90 km (channels 2, 3 and 4), as below this the measurements are complicated by clouds, and above it the theory loses its validity, owing to the weakening of the basic-state zonal wind and the importance of radiative damping. Vertical resolution is limited by the 10-km width of the weighting functions—a fact of great importance in interpreting any theoretical results. Hence, to compare theory and observation, we have used the tides predicted by the model to generate synthetic radiances, and have compared these with the measurements. Orbiter Infrared Radiometer radiances for each channel, accumulated over 72 days, have been binned in a solar-fixed, latitude-longitude, zenith-angle grid¹. Figure 1 displays channel 2, 3 and 4 semidiurnal amplitudes and phases, derived from a Fourier analysis of this grid, as a function of latitude for two zenith angles. Beyond 65°N, strong short and long term variations in atmospheric structure may introduce wavenumber-two features in the time-averaged radiances, whose interpretation as tides is therefore questionable^{1,5}.

Several features appear in the data: (1) The phase increases with height. (2) The amplitude in channel 3 is essentially constant in the meridional direction between 0° and 40°, and then decreases strongly. Similar, though less dramatic, behaviour is seen in channels 2 and 4, which are somewhat more strongly peaked in the tropics. (3) The largest amplitudes are those in channel 3, although classical tidal theory predicts a strong growth of amplitude with height. (4) There is an increase of phase with latitude in channels 2 and 3.

In the Venus mesosphere, the dominant zonal flow is the 'four-day wind'⁶, an extremely strong super-rotation. Therefore, unlike the terrestrial and martian cases, the assumption of an atmospheric mean state represented by a spatially constant angular velocity Ω in inertial space is unsuitable. As a result, 'classical' tidal theory⁷ is not applicable: in particular, the linear tidal equations are no longer separable, and it is in general necessary to resort to multi-dimensional numerical methods for their solution⁸. In the special case, however, where the zonal wind is a slowly varying function of height, an approximation

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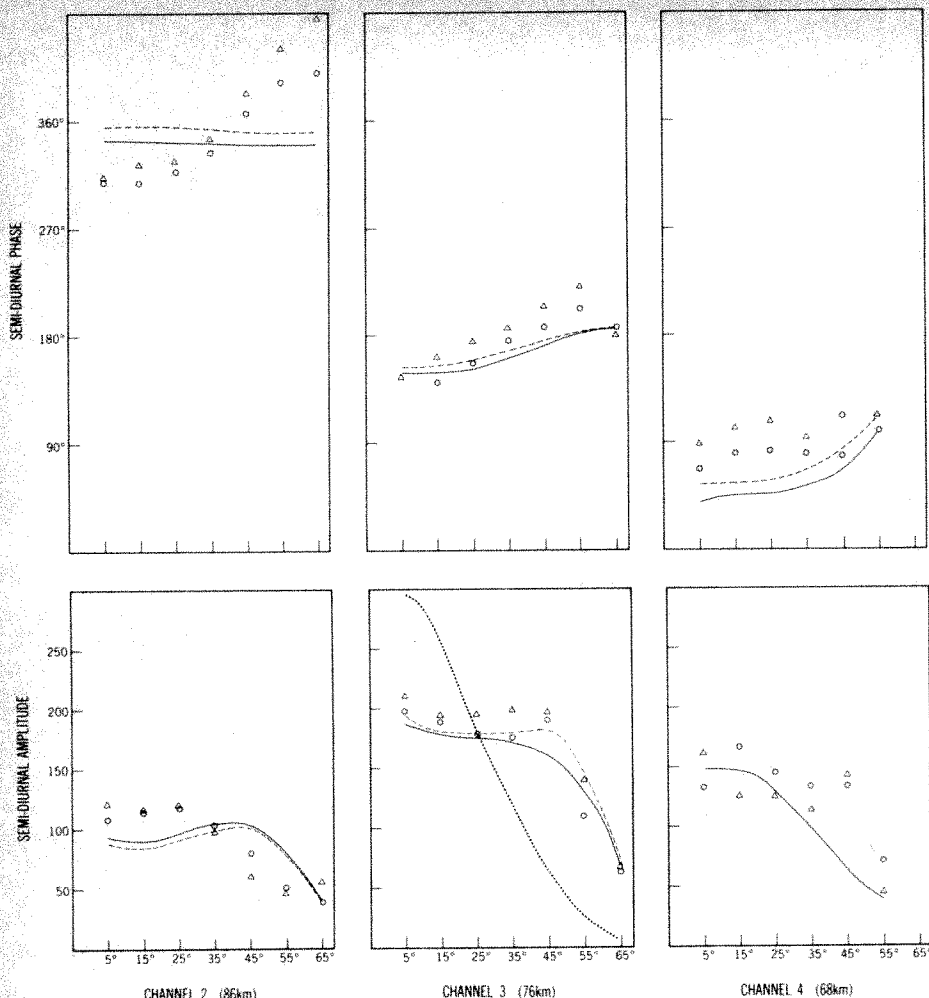


Fig. 1 Observed and predicted semidiurnal radiances. The observed mean amplitudes and phases in each of channels 2, 3 and 4 are shown for two different zenith angles (41° , $\circ$; 57° , Δ). The theoretical predictions for these two zenith angles are the solid and broken line curves respectively. The amplitudes are in units of $\text{nW cm}^{-2} \text{sr}^{-1} \text{cm}$, and the phase convention is that of ref. (1). This is a representative sample of the information available for seven different zenith angles. As explained in the text, poleward of about 65° , the observations are ambiguous, and are therefore not shown. The dotted line in the amplitude diagram for channel 3 shows the meridional structure the tide would have for solid-body rotation.

can be used to preserve much of the simplicity of the classical case (S. B. Fels, unpublished work). This method, which is similar to the Wentzel-Kramers-Brillouin-like techniques developed by others^{9,10}, leads to the description of the tidal geopotential height field $\Phi'(\phi, z, \mu)$ as

$$\Phi' = e^{(2i\phi + z/2H)} \sum_n f_n(z) Y_n(\mu, z) \quad (1)$$

Here $z = -H \log(P/P_0)$, H is a reference scale height calculated at temperature T_0 , ϕ is the longitude, P the pressure, P_0 the surface pressure, and μ the sine of the latitude. The partial differential equation governing Φ' can then be treated as though it were separable, with no coupling between the various modes.

The horizontal structure functions Y_n are generalizations of the usual Hough functions, and satisfy the eigenvalue equation

$$\frac{1}{\omega(\mu, z)} \left\{ \frac{\partial}{\partial \mu} \left[\frac{1-\mu^2}{D} \frac{\partial}{\partial \mu} - \frac{2\mu}{D} \right] + \frac{1}{D} \left[\left(2\mu - \frac{1-\mu^2}{\omega} \frac{\partial \omega}{\partial \mu} \right) \frac{\partial}{\partial \mu} - \frac{4}{1-\mu^2} \right] \right\} Y_n = -\varepsilon_n^2(z) Y_n \quad (2)$$

at each height z . Note that $\omega(\mu, z) = \Omega(\mu, z)/\Omega(0, z)$. By assumption,

$$\frac{dY_n}{dz} / Y_n \ll \frac{df_n}{dz} / f_n$$

The vertical structure functions satisfy the simple second-order ordinary differential equation

$$\left(\frac{\partial}{\partial z} - \frac{1}{2H} \right) \left[\frac{1}{N^2} \left(\frac{\partial}{\partial z} + \frac{1}{2H} \right) f_n \right] + \frac{\varepsilon_n^2}{a^2 \Omega_c^2(z)} f_n = \frac{R}{2iH} \left(\frac{\partial}{\partial z} - \frac{1}{H} \right) \frac{1}{N^2} \int d\mu Y_n(\mu, z) J(\mu, z) / \Omega(\mu, z) \quad (3)$$

Here $N(z)$ is $T_0/T(z)$ times the Brunt-Vaisala frequency, which is based on the observed equatorial static stability, and independent of latitude; Ω_c , the mean-state angular velocity at the equator; a , the planetary radius; R , the reduced gas constant; J , the tidal heating rate. Below 85 km, the contribution of the higher modes to the sum in equation (1) is small, and so for simplicity we will retain only the gravest mode in our numeric calculations.

The meridional structure of Y_n depends strongly on that of the normalized angular velocity $\omega(\mu, z)$, and so contains information about the behaviour of U in the horizontal direction. The vertical wavelength of the tide is roughly $\Omega_c a / N \varepsilon_n$, so that this quantity provides a measure of the meridionally averaged zonal velocity. In the models we have examined, the gravest semidiurnal tide has a vertical wavelength of 25 km or less in the region of interest.

The tidal heating J is derived by Fourier analysis from the results of a detailed radiative model of the Venus mesosphere. This model, whose opacity sources include CO_2 , SO_2 and H_2 as well as cloud particles, was constrained by use of Pioneer Venus observations. The results show that there is an important region of excitation near 64 km, due to the strong absorption of ultraviolet and near-infrared solar radiation by the cloud particles in that region (Fig. 2). A less important heating region due to the near infrared CO_2 bands appears above 80 km.

Damping of the tides by thermal infrared transfer becomes important whenever the radiative decay time $\tau_{\text{rad}} < \Omega^{-1}$. This is generally true above 83 km in our models and has been taken into account, by modifying equations (2) and (3), to allow for the effects of newtonian cooling. The required relaxation rates (Fig. 2) were taken from ref. (11), and are those appropriate for a disturbance of vertical wavelength ~ 25 km. (They are approximately twice those appropriate for an infinite wavelength case.)

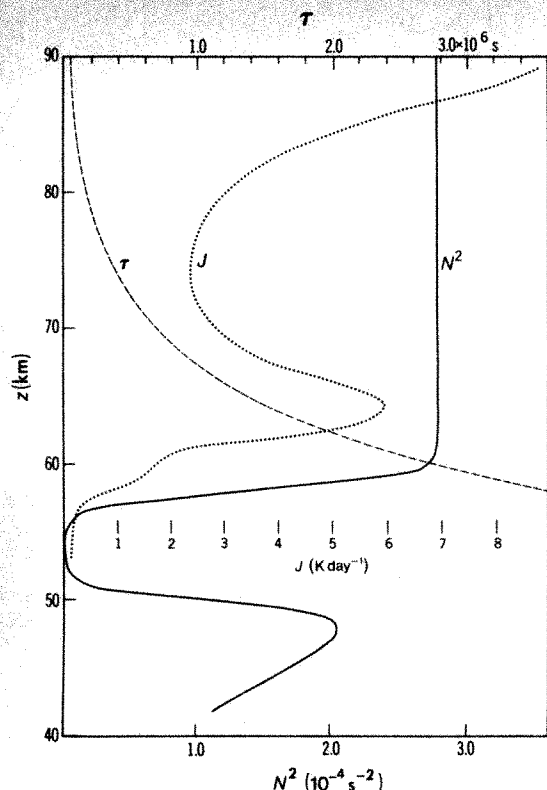


Fig. 2 Vertical structure of the model atmosphere from 40–90 km. The solid line is N^2 , the Brunt-Vaisala frequency squared, in units of s^{-2} (bottom scale); the dotted line is J , the semidiurnal component of the solar heating function at the Equator in degrees K per Earth day (middle scale); the chained line is τ_{rad} , the radiative damping time in seconds (upper scale).

Although the approximations used in reaching this formulation may not be strictly valid, the extreme simplicity of the method and the ease with which it allows for interpretation make it very useful in preliminary studies like this. The simplicity of the theoretical model makes it possible to test many different forms for U . We always demand that U , at 60 km, be close to the form deduced from Pioneer Venus ultraviolet cloud observations¹², which imply an equatorial velocity of $\sim 100 \text{ ms}^{-1}$, and a rotation profile which is close to solid-body.

Figure 3 shows the structure of U that best fits the tidal observations; the resulting predictions are given in Fig. 1. The agreement is seen to be very good in general. The most noticeable errors occur in channel 2, where the latitudinal structure of the amplitude and phase is not well reproduced. This is probably owing to the inclusion of only one mode, whose structure does not match that of the local heating at 90 km. At this level and above, $\Omega\tau_{\text{rad}}$ is small, and the thermal structure depends largely on the local heating. The errors in the amplitude and phase in channel 4 are possibly owing to incorrect specification of the details of the heating function J .

The following considerations support our choice of U :

- (1) Solid-body rotation results in tidal fields whose meridional structure is much too strongly peaked near the equator. To match the observations, especially those of channel 3, it is necessary that Ω be much larger at 50° than at the equator. This has the additional desirable effect of increasing the vertical wavelength of the tide between 70 and 86 km. Without this, and without the region of large U between 67 and 71 km, the phases of channels 2 and 3 would be much too large.

- (2) The existence of the overall $e^{z/2H}$ term makes it extremely difficult to keep the channel 2 amplitude below that of channel 3, unless U decreases dramatically with height above 78 km. This has the effect of reducing the parameter $\Omega\tau_{\text{rad}}$ so that damping becomes effective. Also it reduces the vertical

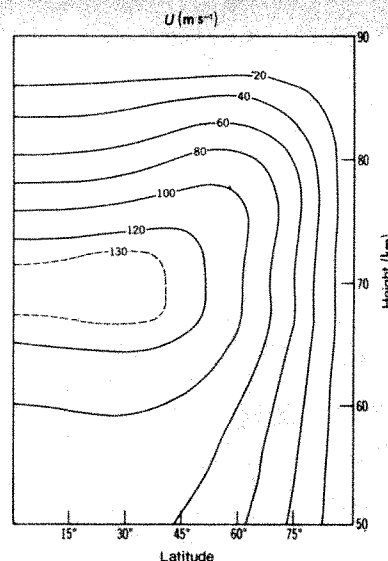


Fig. 3 Vertical and meridional structure of $U(\theta, z)$, the zonal mean wind used in the theoretical tidal model.

wavelength near 88 km, so that the averaging effect due to the thickness of the channel 2 weighting function becomes important. The same conclusion has been reached independently by Pechmann⁸.

The meridional variation in the phase of the tide is largely owing to the effect of radiative damping which destroys the modal character of the horizontal eigenfunction, and allows some meridional propagation to occur.

Whereas it is possible to find other mean-state U structures which give equally good tidal predictions, they all share the chief characteristics of that displayed here. They are also in general agreement with those deduced by Limaye (S. S. Limaye, paper presented at Venus Int. Ref. Atmos. Workshop, Hamburg, 1983) on the basis of cyclostrophic balance with the measured zonal mean temperature, but differ in that the balanced winds are considerably weaker in the tropics than are ours. However, such diagnostic calculations are not without problems¹³, and particularly so in the tropics. The tidal calculations, on the other hand, do not share this deficiency. Indeed, the tidal temperature perturbations based on Limaye's U disagree with observations in two respects: they are too strongly peaked at mid-latitudes and their channel 2 and 3 phases are too large in the tropics by 50° . This is true even when U in the tropics is held fixed at its value at 30° . If, as Limaye suggests, U decreases towards the equator, these failures would be even more pronounced. If, on the other hand, U were to increase equatorward of 30° (S. S. Limaye and A. J. Kliore, preprint), the agreement with observed tides would be better.

The form of U which we propose is not only broadly consistent with observations^{13,14}, but also with theoretical speculation on the role the tides themselves have in decelerating the mean flow above the forcing region¹⁵. In addition, conclusions quite similar to ours have recently been reached by P. J. Valdes (personal communication) using a two-dimensional numerical tidal model. Although this increases confidence in our results, their confirmation must await further observations.

This work was begun while one of us (S.B.F.) was a visitor in the Department of Atmospheric Physics, Oxford University. We thank Drs D. G. Andrews and F. W. Taylor for useful discussions, and Mr P. J. Valdes for showing us his calculations before their publication.

Received 15 June; accepted 25 September 1984.

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Comparative utility of microwave and shortwave satellite data for all-weather charting of snow cover

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Charting snow cover by shortwave imaging requires visual analysis which is complicated by cloud coverage and poor surface illumination^{1,2}. Microwaves, however, being almost unaffected by clouds and independent of solar illumination, are potentially useful for monitoring the extent and variation in snow cover, in climatological and hydrological studies. Data from spaceborne passive microwave sensors have previously been used to chart regional overland snow cover³⁻⁵; but charting hemispherical snow coverage was not feasible until high spatial resolution and multiple channels were combined in the Scanning Multichannel Microwave Radiometer (SMMR) launched in 1978 on the Nimbus-7 satellite. Here we compare SMMR data with shortwave images obtained over Asia to justify further use of microwave sensors in automated charting of seasonal snow cover under all weather conditions. Agreement between the two methods is found in ~75% of the tested grid points.

Microwave radiation emitted from the Earth's surface is detected by the Scanning Multichannel Microwave Radiometer at five frequencies, in two polarization planes. Radiance in the microwave region is usually expressed by the apparent brightness temperature $T_b = eT$, where T is the physical temperature of the emitting layer and e is its emissivity ($e \leq 1$). The emission comes from throughout the snow pack as well as from the underlying ground. The discrimination of surface types is possible mainly because of differences in emissivity. Figure 1 shows some examples. Whereas a snow-free soil shows little variation in emissivity over the whole frequency range, the emissivity of dry snow decreases with increasing frequency. This decrease depends on the thickness of the scattering layer and gives an indication of the water equivalent of the dry snow. The microwave penetration depth of wet snow is small owing to the high dielectric losses of water, so that the microwave signatures of dry and wet snow differ significantly. Regions undergoing a daily freeze-thaw cycle, commonly observed over melting snow fields in spring, can be recognized by comparing day and night microwave signals. However, a snow layer which is continually wet cannot be distinguished from snow-free ground by our algorithm, which may underestimate the snow fields undergoing heavy melt⁶.

The preceding snow cover parameters can best be obtained by an analysis of the 18 and 37 GHz horizontally polarized

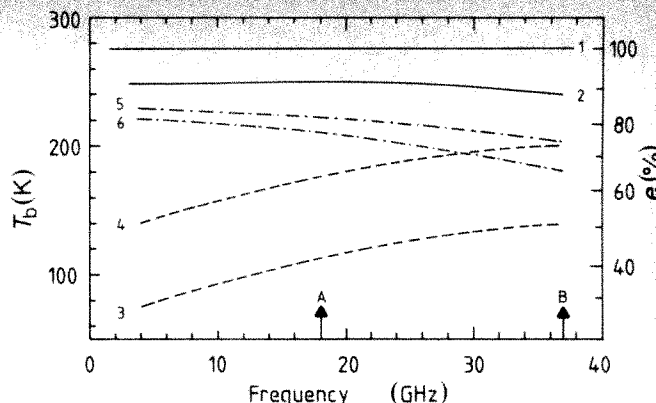


Fig. 1 Brightness temperatures (T_b) and emissivity (e) at microwave frequencies for: (1) an ideal black-body (assumed physical temperature of 273 K); (2) snow-free ground; (3) and (4) a flat sea surface, (3) horizontal and (4) vertical (physical temperature $\sim 10^\circ\text{C}$, incident angle $\sim 50^\circ$); (5) snow-covered ground or old sea ice and (6) with increasing snow depth (horizontal). Curves (2), (5) and (6) are typical values obtained from ground-based microwave sensors¹⁵ and curves (3) and (4) are based on theoretical calculations and measurements¹⁶. A and B mark the SMMR frequencies used for snow retrieval.

SMMR channels⁷, with spatial resolution of 60×60 km and 30×30 km, respectively. The data from three days and nights, during a five-day interval, give complete coverage for areas polewards of 25° latitude. Because of satellite power limitations data are recorded only on alternate days. The fully automated analysis consists of reading the data from tape, applying the retrieval algorithm and displaying the data on a colour screen. A pixel is classified as covered with dry snow when the difference in brightness temperature between the 18 and 37 GHz data is less than a prescribed threshold determined empirically from tests with ground data from throughout the middle and high latitudes of the Northern Hemisphere. Additional thresholds enable further differentiation between cover of more than 10 cm and thin or patchy snow. The result shows the maximum extent of snow cover over the five day period. The algorithm is described in more detail by Kunzi *et al.*⁷.

To assess the accuracy of the microwave analysis, we compared the SMMR data with snow charts based on shortwave imagery from March 1979. The central Asian region was selected because its terrain and vegetation are highly variable, because high-quality cloud-free imagery in the visible is common at this time of the year and because it is climatologically important⁸⁻¹⁰. As only a limited number of ground stations in this region report snow, only remote techniques can be used.

Snow was charted from the shortwave imagery collected daily by a polar-orbiting Defense Meteorological Satellite Program sensor with a spectral range of $0.4\text{--}1.1 \mu\text{m}$ and subtrack resolution of 2.8 km (ref. 11). Imagery was available on ungridded positive film transparencies on a scale of 1:15 million. A trained observer examined visually the transparencies from consecutive days and plotted the snow coverage on a gridded base-chart. Snow cover was differentiated from clouds and snow-free land by its characteristic surface brightness, textures and persistence^{12,13}. Two classes, full and partial cover, were distinguished. Areas where persistent clouds completely obscured the surface throughout the study period were charted as such and excluded from the analysis.

Microwave and shortwave charts for the intervals 11-15 March and 21-25 March 1979 were compared. No adjustment was made to improve the apparent agreement. Figure 2 shows the charts for 11-15 March. Grid points at the intersections of every 2.5° of longitude (50°E – 140°E) and 1° of latitude (28°N – 60°N) were categorized according to charted microwave and

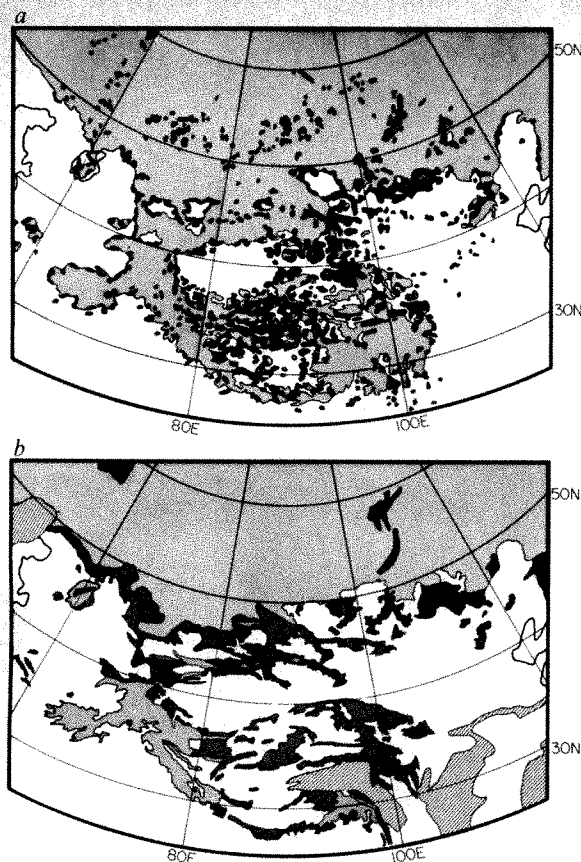


Fig. 2 Scanning Multichannel Microwave Radiometer (a) and Defense Meteorological Satellite Program (b) derived Asian snow cover for 11-15 March 1979. Fully snow-covered ground is shown in light stipple, partially covered is in dark stipple and snow-free is blank. Persistent cloudiness in b is hatched.

shortwave conditions. The results are shown in Table 1. Only 3.5% and 4.5% of the 924 gridpoints analysed on the 11-15 March and 21-25 March charts, respectively, were excluded because of persistent cloud cover. However, in other seasons clouds are more frequent in this region. Areas classified on the microwave charts as having dry snow deeper than 10 cm were assumed to be fully covered.

There is reasonable agreement between the microwave and shortwave classifications of surface conditions. Complete agreement is found over 76.4% (11-15 March) and 73.5% (21-25 March) of the area, whereas only 3.9% and 8.6% show full snow cover in one chart and no snow in the other (Table 1).

The best agreement between the charts occurs in areas where shortwave charts show full cover and where microwave charts show snow-free ground. Microwave charts tend to indicate more snow than the visual analysis. This is true for situations where partially covered areas in microwave analyses are classified as snow free in shortwave charts, and where full cover on microwave charts is seen as partial cover in the shortwave. Disagreement is most frequently observed over the Tibetan plateau, where snow-free ground intersperses the snow-capped mountains. Possible reasons for this are: (1) the much higher spatial resolution of the shortwave chart enables the recognition of individual snow patches covering only a small portion of an area, whereas microwave signals from these snow patches are combined with those of the adjacent snow-free ground and the area as a whole is classified as partially snow-covered; (2) imagery employed in this study may reflect time differences of up to 4 days, so that the snow observed in one set may have been absent in the other; (3) night-time dry-sand microwave

Table 1 Comparison of microwave and shortwave-based snow classification in per cent of the total number of gridpoints

11-15 March 1979

Shortwave classification	Microwave classification			Sum shortwave
	Fully snow-covered	Partially covered	Snow-free	
Fully snow-covered	39.7	2.5	0.5	42.7%
Partially covered	8.4	3.5	2.6	14.5%
Snow-free	3.4	6.2	33.2	42.8%
Sum microwave	51.5%	12.2%	36.3%	

21-25 March 1979

Shortwave classification	Microwave classification			Sum shortwave
	Fully snow-covered	Partially covered	Snow-free	
Fully snow-covered	35.3	2.8	2.4	40.5%
Partially covered	4.5	2.7	3.1	10.4%
Snow free	6.2	7.4	35.5	49.1%
Sum microwave	46.0%	12.9%	41.0%	

signatures are similar to those of thin dry-snow layers because the frequency-dependent penetration depth in dry sand is approximately several wavelengths and vertical temperature gradients in the sand on clear nights are strong.

The Scanning Multichannel Microwave Radiometer has now provided satellite data for almost six consecutive winter seasons in the Northern Hemisphere. Additional tests with shortwave satellite images and, where available, correlative ground data will further our understanding of the microwave data set. At present the multispectral microwave method appears to be very promising for automated large-scale charting of snow. This will prove invaluable, especially in autumn and early winter when snow fields develop in poorly illuminated areas under heavy cloud cover, making conventional charting difficult and inaccurate¹⁴. However, full substitution of shortwave snow-cover analysis by microwave mapping will not be possible in climate studies as one of the most critical snow parameters, its high albedo, cannot be assessed using microwaves.

We thank Michael Fried for assistance with the shortwave analysis. This project was supported by the Swiss National Science Foundation, the Austrian Fonds zur Förderung der Wissenschaftlichen Forschung and NSF contract ATM82-00863. This is Lamont-Doherty Geological Observatory contribution no. 3730.

Received 4 June; accepted 18 September 1984.

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Surface faulting in the southern Italian Campania-Basilicata earthquake of 23 November 1980

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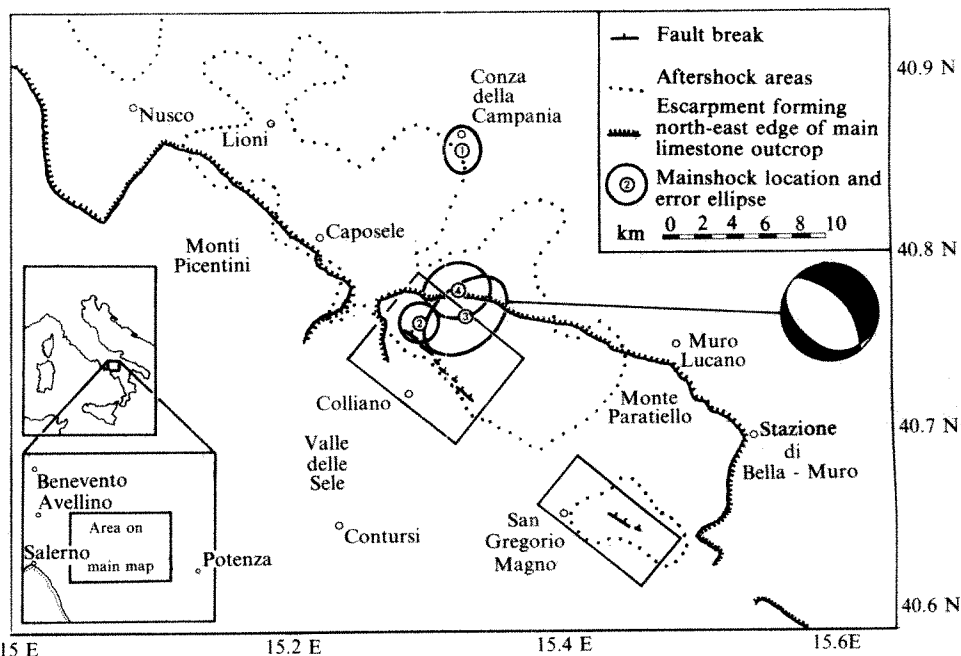
The Campania-Basilicata earthquake of 23 November 1980 was the largest ($M_s = 6.9$) and most destructive earthquake that has occurred in this region of southern Italy for over 100 years, killing more than 3,000 people. The consensus among seismologists, geologists and earthquake engineers who have studied¹⁻¹⁰ this event is, in keeping with previous Italian earthquakes, that it did not produce faulting at the surface. This has led to the establishment of a number of models^{2,3,11-13} requiring an unusual tectonic setting for the southern Apennines. However, on a recent visit to the epicentral region of the earthquake (May 1984), we discovered more than 10 km of surface faulting that is consistent with focal mechanisms for this event, based on first motion polarities^{2,4,5,12} and long period waveform studies¹⁴⁻¹⁶, which show that it involved normal faulting with one nodal plane dipping at $\sim 60^\circ$ towards the north-east. Our observations of this—the first well-documented earthquake fault break to be reported in Italy—suggest that the deformation associated with earthquakes in the southern Apennines takes place in the upper 10–15 km of the Earth's crust on steep planar normal faults which break the surface, as is widely observed in other areas of active extension outside Italy.

We found most of the fault break at altitudes of $> 1,300$ m in remote limestone mountains, covered with dense beech forest,

far from habitation or lines of communication. This area remained snow-covered until the spring following the earthquake and, consequently, the fault break escaped detection at the time of the earthquake. In the spring of 1981 the area was visited by many scientists (for example, the authors of refs 6–10, 17). Geologists from CNEN (Comitato Nazionale dell'Energia Nucleare) photographed parts of the break and, having interpreted it as a landslide, sent us their photographs, prompting this subsequent investigation. Our observations, when combined with the seismological evidence discussed below, strongly suggest that these surface breaks are of primary tectonic origin, and are not superficial effects.

Figure 1 shows the two separate segments of fault break identified. The longer segment is situated near the north-west end of the Monte Marzano-Monte Paratiello massif, to the east of the upper Sele valley (Figs 1, 2a), and consists of several strands each striking at approximately 300° and downthrown to the north-east. It extends for ~ 10 km from Piano Acquafredda, southeastwards to Piano Neurale. Between the strands it steps to the right, making its overall strike closer to 320° . The vertical displacement across the break is ~ 1 m in the middle of each strand, but smaller towards the ends. Much of this segment was well-known to local shepherds and labourers, who confirmed that it did not exist before the 1980 earthquake. Observations of a part < 2 km long, around the Piano di Pecore, have already been reported^{9,10}. These authors believed the break to be a secondary feature due to slipping or subsidence of superficial sediments. However, the individual strands of the break are straight, cutting across the topography. The overall trend of this segment is linear and crosses from the northeastern side of the high topography to the southwestern side near the col of Piano di Pecore. South-east of locality 5 in Fig. 2a, the uphill side of

Fig. 1 Positions of the observed fault break of the 1980 earthquake in relation to mainshock and aftershock locations. The focal mechanism for the mainshock (lower focal hemisphere, equal area projection, compressional quadrants shaded) is by Westaway and is based on first motion polarities observed at long period stations of the World-Wide Standard Seismograph Network. The north-east dipping nodal plane (dip 60° , strike 315°) is well constrained, and the other nodal plane (dip 30° , strike 125°) has been drawn to give the mechanism the same rake as in the observed fault break (80°), assuming the north-east dipping plane is the fault plane. This second nodal plane is not well constrained; its strike can be varied by up to 20° without introducing an inconsistency with any observed first motion polarities. Mainshock location 1, constrained at 0 km depth, is that of the ISC (International Seismological Centre), obtained from arrival times at 506 teleseismic stations. Location 2, at 16 ± 1 km depth, by del Pezzo *et al.*¹², was obtained using 11 Italian regional stations within 200 km of the epicentre. All the stations used lie in the north-west or south-east quadrants, and this inadequate azimuthal coverage makes the location of the epicentre potentially unreliable. Location 3, at 16 ± 2 km depth, was obtained by Westaway using the location program HYP071²⁵ with data from 49 regional stations within 400 km. Because Balkan and Sicilian stations are now included, the azimuthal coverage is much better and the horizontal coordinates are better determined, but as with location 2, the focal depth is not well determined, even though the nominal uncertainty in depth, calculated by the location program, is small in both cases. Location 4, at 15 ± 8 km depth, was obtained by Westaway by relocating the mainshock relative to a large aftershock²⁶, the location of which is well determined by the temporary local seismograph network. Sixty-three stations were used in this relative relocation. Depth is not well determined in any of the locations 1–4, although preliminary waveform modelling⁴ and aftershock locations suggest that the mainshock nucleated somewhere in the depth range of 10–15 km. Although our preferred location is 4, numbers 2, 3 and 4 are all in about the same place and are certainly expected to be more reliable than the ISC location. Although the reported errors for the ISC location are small, it is systematically misplaced to the north of the true location, a feature common to many teleseismic locations in the Mediterranean area, for which most of the stations recording any earthquake are to the north. The assumption that the ISC location is correct has led to the suggestion⁴ that the 1980 mainshock was caused by the motion of a fault whose surface expression is the large topographic escarpment extending south-east from Laviano past Muro Lucano as far as Bella-Muro railway station. This escarpment forms the northeastern edge of the Monte Marzano-Monte Paratiello limestone outcrop. The observations presented here do not support this suggestion.



the rupture is downthrown. Along the break there are frequent exposures of a limestone fault surface with a consistent dip and strike, which at several places (for example, localities 1, 3 and 6 in Fig. 2a) appears to have moved in the past. The overall pattern of successive strands stepping to the right implies that the normal fault has a small component of left-lateral strike slip, and agrees with the sense of the motion at the one place where it could be directly measured (locality 7 in Fig. 2a). These observations strongly suggest that this segment is a fault break, not a landslide, and, as the largest aftershock was of only $m_b = 4.8$ ($M_s = 4.4$), the fault probably moved in the mainshock itself.

Figure 1 compares the position of this break with aftershock locations obtained from a network of more than 30 temporary seismographs installed in the epicentral area following the mainshock^{3,12,18,19}. In the centre of this network, aftershocks could be located with an accuracy of $\sim \pm 1$ km in horizontal coordinates and $\sim \pm 2$ km in depth. Most aftershocks are shallower than 12 km, with some as deep as 15 km. A clear feature of Fig. 1 is the cutoff in aftershock locations within 1 km of the position of the north-west segment of fault break. Very few aftershocks occurred south-west of this.

The second segment of fault break, with a throw of ~ 0.5 m, was found in the Pantano di San Gregorio about 5 km east of the village of San Gregorio Magno, situated about 16 km south-east of Piano di Pecore (Fig. 2b). Although only 3 km long, this

south-east segment has a similar strike to and lines up along strike with the north-west segment. A cluster of large aftershocks (Fig. 1) has been located under this segment, although, being situated near the edge of the temporary seismograph network, their locations are less reliable than those further north-west. This evidence and that of a limestone fault surface observed at locality 9 (Fig. 2b) suggest a primary tectonic origin for the surface displacements in this segment also. Moreover, as discussed below, the north-west segment cannot, by itself, account for all the faulting in the mainshock.

The displacements measured in the field are consistent with the observed drop in elevation obtained by releveling¹ in 1981 along a road running approximately parallel to but 8–12 km north-east of a line joining the two observed segments of fault break. Since the first levelling in 1959, the elevation of the road between Bella-Muro railway station (Fig. 1) and a point 6 km north of Laviano (Fig. 2a) had decreased by 20 cm in the east and up to 70 cm in the west, presumably as a result of the 1980 earthquake.

Figure 1 shows relevant seismological observations, including a focal mechanism and four possible epicentral locations for the mainshock, of which location 4 is probably the most reliable. A planar downward projection of the surface faulting (mean dip 62° north-east, strike 320°) passes beneath epicentre 4 at a depth of 10 km with an orientation almost the same as that of

Fig. 2 Detailed maps of the location: *a*, of the northwestern (Piano di Pecore) segment of fault break; *b*, of the southeastern (San Gregorio) segment. *a*, Between Piano Acquafredda and Piano di Pecore, the northwestern segment runs for 6 km along a northeast-facing escarpment, which rises to the summits of Monte Valva and Monte Marzano and which is mapped as a fault offsetting Cretaceous limestone. This escarpment continues 4 km further north-west to the edge of the limestone outcrop, and, though it is possible that faulting continued along this section also, it could not be traced because of the rugged topography, the thickness of scrub, and the debris from landslides covering much of this area. At locality 1, close to Piano Acquafredda, the break cuts a little-used forest track, and is visible in the track cutting. It is clear that at this point the same fault plane has moved in the past, as alluvium on the north-east side has been faulted down against limestone. The break can be followed through the forest to locality 2, where a 100-m long continuous limestone face (dip 63° , strike 304° , throw 1 m) with a smooth surface of cemented fault breccia is exposed. At locality 3, the break follows an abandoned forest track which appears to have been built along a ledge created by past motion of the same feature. At 4, one of the localities first noted in 1981^{9,10}, it descends the mountain side and cuts a road, originally forming a step with an offset of 1 m, now filled in, but still clearly visible. Close to the road the break resembles a landslide, but to the north-west it follows a uniform strike (310°) for 1 km, with frequent exposed planar surfaces of polished limestone breccia along it. To the south-east it is visible as a 0.5-m high scarp, downthrown to the north-east, and crossing Piano di Pecore; a small, flat, alluvium-filled basin on the northeast side of the escarpment. This basin is ponded by the escarpment and drains through it to the south-west. Further south-east, the break is again visible close to the top of the ridge separating Piano di Pecore from Piano Neurale. Along most of this section, the downthrown side, still to the north-east, is uphill. At locality 5, there is another exposure, 50 m long, of a planar limestone surface (dip 62° , strike 306° , throw 0.8 m) covered with fault breccia. At locality 6, the break occurs on the southwest side of a 100-m long gully, which a stream has eroded to a depth of 4 m into the limestone, separating it from the uphill slope to the north-east. This again suggests that the same feature has moved previously, before the 1980 earthquake. Further south-east, the break descends the south-west face of the ridge before finally dying out close to Piano Neurale. An extensive search further south-east failed to yield any evidence of continuation. At locality 7 (Figs 3, 4) the fault break occurred on a polished planar limestone surface with strike 298° and dip 60° . The base of this surface was stained by alluvium and indicates a throw of about 0.5 m during the earthquake. Striations on the stained surface indicate a slip vector with rake 80° north-west, that is, with a small (10°) component of left lateral motion. This is consistent with the right-stepping nature of the entire north-west segment, a feature observed in many other earthquakes, for example, ref. 27. *b*, Photographs¹⁷ of the southeastern segment in the Pantano di San Gregorio, taken in 1981, showed a surface break on this plain at locality 8 and, although the area has subsequently been ploughed over, the vertical offset of ~ 0.5 m, which strikes at 306° and is downthrown to the north-east, is still visible west of the road running around the south side of the plain. On the other side of this road, at locality 9, the rupture follows a polished limestone fault surface exposed at the edge of the plain (dip 68° , strike 298° , throw 0.4 m). This rupture could be traced for ~ 500 m before it became lost in the scrub. This segment may have extended further north-west of the Pantano di San Gregorio, but this could not be verified as the north-west edges of the plain, and the surrounding hillsides, have been covered with rubble dumped from the shattered village of San Gregorio Magno.

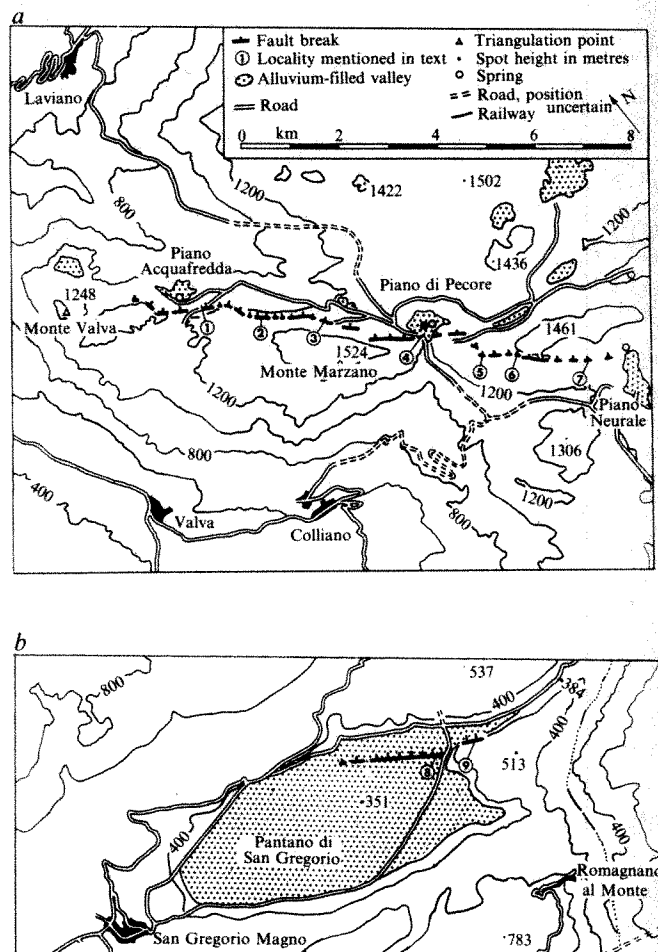




Fig. 3 The fault break looking north-west from locality 7. This is one of the few places where the surface break crosses open ground.

the north-east dipping nodal plane in the focal mechanism of the mainshock (dip 60° north-east, strike 315°). Our observations are thus consistent with a planar fault geometry and a nucleation depth of 10 km for the mainshock. This depth is within the uncertainty in depth for location 4. Such a planar geometry is seen for other active normal faults whose source parameters are well resolved²⁰.

However, it is clear that the observed break on the north-west fault segment cannot account for all the motion that took place during the mainshock. The seismic moment of the mainshock, obtained by a moment-tensor inversion of long period body and surface waves¹⁴, is 3×10^{19} N m. Assuming a displacement of 1 m on a fault that extends 10 km horizontally, and from the surface to 15 km depth at a dip of 60° (corresponding to the north-west fault segment), the moment released would be only 6×10^{18} N m in rocks with a shear modulus of 3×10^{10} N m⁻². Additional faulting must have occurred elsewhere, and in the absence of any other information, the clusters of aftershocks shown in Fig. 1 may indicate further segments where faulting occurred during the mainshock. The ends of these segments occur at places where major structural discontinuities, such as that marked by the Sele valley²¹, intersect the structural and topographic trend. To account for the moment estimated from moment-tensor inversion, the amount of slip on each of these segments would need to be less than 1 m. This type of segmented fault geometry, controlled by structural discontinuities on a scale of ~ 10 km, appears to be common for large crustal earthquakes (see, for example, ref. 22).

The 1980 earthquake was the largest to have occurred in this region of southern Italy since the event of 16 December 1857, which occurred about 80 km further south-east and was studied by Mallet²³. He reported no surface faulting associated with the 1857 event, but he was in the epicentral area only between

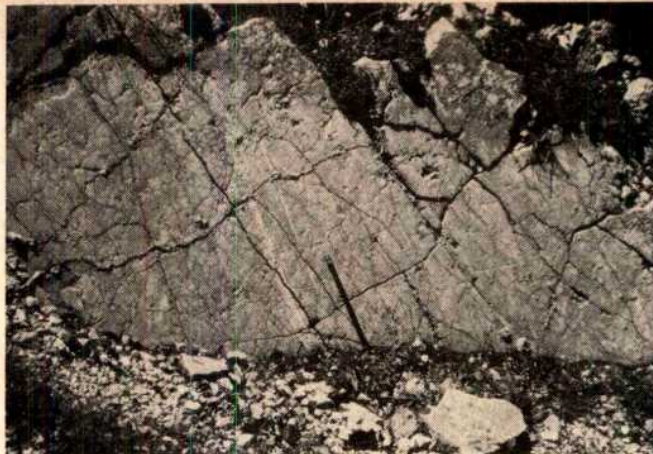


Fig. 4 Close up photograph, facing south-west, of the fresh striations (parallel to the pencil), stained by alluvium, observed on the fault at locality 7, indicating a small left-lateral component of strike slip.

February and March 1858, and was unable to examine the high mountains then covered in snow. Consequently, surface faulting in the 1857 event may have occurred, but remained undetected.

Between 1857 and 1980, two earthquakes comparable in size to the 1980 event occurred in the same area. Neither of these events on 23 July 1930 ($M_s = 6.5$)⁵ and 21 August 1962 ($M_s = 6.2$)²⁴, was well recorded, and the uncertainty in the hypocentral location in both cases is much greater than for the 1980 event. The reported depths of 40 km (ref. 24) and 28 km (ref. 2) for the 1962 event are unreliable, particularly as the closest instrument to record this event unambiguously was more than 500 km distant. Thus, the 1980 event provides the main body of reliable information on which to base an understanding of the neotectonics of the southern Apennines; the evidence presented here suggests that deformation in this region occurs in the brittle part of the upper crust, on steep planar normal faults which break the surface.

We thank the geologists and seismologists of CNEN and the University of Naples, especially Roberto Scarpa, for assistance and helpful discussions; the NERC for financial support; Shell International for a research scholarship for R. W., and a travel grant; Kathryn Garson for assistance in the field; G. King for a preprint of ref. 3; Helen Anderson and J. Ansell for suggesting improvements to the original manuscript. Cambridge University Earth Sciences publication no. 524.

Received 22 June; accepted 25 October 1984.

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A Grenville Sm-Nd age for the Glenelg eclogite in north-west Scotland

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Eclogite, a garnet-pyroxene rock with basaltic chemistry, is a product of unusually high-pressure metamorphism. In Scotland, it occurs within the Caledonian orogenic belt close to the Moine thrust, where it forms partially amphibolitized, easterly-dipping layers, associated with metasediments in the eastern Glenelg Lewisian inlier¹⁻⁴. In spite of its potential importance for an understanding of the geological history of north-west Scotland, its age has never been established more precisely than 'probably Proterozoic'⁴. A K-Ar omphacite age of 1,515 Myr⁵ is rendered unreliable by the possible presence of excess argon. We report here the results of an attempt to date the eclogite metamorphism by the Sm-Nd method. Two samples of eclogite yielded mineral isochrons of just over 1,000 Myr. The results represent clear evidence of Grenville metamorphism in Scotland, and imply close proximity of its front to the Moine thrust near Glenelg. Also, as the local Moine cover has not apparently reached eclogite facies, it was presumably deposited on an eroded Grenville basement.

The two samples selected for analysis, S215 and S274, are fresh, coarse-grained (>2 mm), granoblastic rocks: S215 consists of pale green, omphacitic pyroxene, pink garnet and some interstitial pargasite; S274 has more deeply coloured omphacite and garnet, and contains much quartz. Both rocks carry accessory rutile and show slight breakdown of omphacite to pyroxene-plagioclase symplectite. Mineral and rock compositions are given in Table 1: garnet and omphacite are unzoned; the rocks are meta-tholeiites. The eclogite samples were collected from the tract of country extending south-west from Loch Duich,

where the effects of ?Caledonian amphibolitization are minimal⁴. S215 comes from a thick eclogite layer overlying a spectacular cliff of marble, above Lochan na Beinn Fhada (grid ref. NG 860234); S274 comes from another thick layer at Cruachan Eilgeach (NG 841212).

The results of the analyses are presented in Table 1 and Fig. 1. The mineral-whole rock isochron ages, $1,010 \pm 13$ Myr and $1,082 \pm 24$ Myr ($\lambda^{147}\text{Sm} = 6.54 \times 10^{-12} \text{ yr}^{-1}$, 2σ uncertainty), are interpreted as reflecting the closure of Sm and Nd isotopic exchange between garnet and omphacite during post-metamorphic cooling. The blocking temperature for this system has been estimated as 600 °C (ref. 6); it was probably reached relatively soon after eclogite formation, as the high pressure of metamorphism (>12 kbar, see below) suggests that the crust was unusually thick, and that isostatic recovery would have been

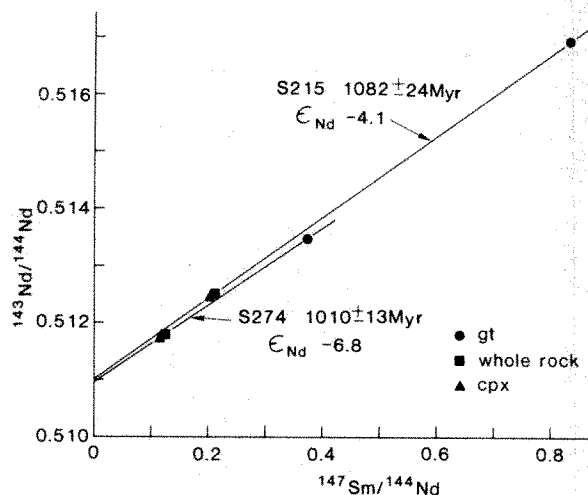


Fig. 1 Sm-Nd mineral-whole-rock isochrons from two samples of Glenelg eclogite. ϵ_{Nd} is the initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratio expressed as the deviation in parts per 10⁴ from the contemporaneous Nd-isotope ratio of the bulk earth.

Table 1 Major and rare earth element data for eclogite samples

	S215 rock	S215 gt	S215 cpx	S274 rock	S274 gt	S274 cpx	S135 rock
SiO ₂	47.33	39.02	53.76	49.33	38.57	53.56	52.46
TiO ₂	1.29	ND	0.28	3.31	0.05	0.28	1.59
Al ₂ O ₃	12.60	22.02	7.65	12.89	21.71	8.67	13.26
Fe ₂ O ₃	13.73	—	2.09	17.89	—	2.87	15.19
FeO	—	22.12	3.75	—	26.57	5.62	—
MnO	0.28	0.64	0.06	0.18	0.53	0.02	0.26
MgO	10.54	8.41	10.39	6.22	5.86	7.98	6.61
CaO	12.93	7.78	17.15	8.83	6.69	14.54	8.79
Na ₂ O	2.53	—	4.45	2.03	—	5.50	1.86
K ₂ O	0.04	—	0.01	0.04	—	—	0.07
Total	101.27	100.00	99.60	100.72	99.98	99.04	100.09
La	—	0.439	2.59	—	12.7	57.1	—
Ce	—	1.18	21.5	—	33.7	106	—
Nd	6.30	1.61	31.6	34.7	24.5	67.0	9.28
Sm	2.10	2.22	10.6	6.91	15.2	12.8	3.16
Eu	—	1.24	2.89	—	7.27	2.98	—
Gd	—	6.76	8.28	—	31.3	8.12	—
Dy	—	11.3	3.46	—	35.5	3.31	—
Er	—	7.01	0.884	—	18.5	1.17	—
Yb	—	6.33	0.467	—	15.0	0.826	—
¹⁴⁷ Sm/ ¹⁴⁴ Nd	0.2015	0.8348	0.2029	0.1206	0.3753	0.1156	0.2059
¹⁴³ Nd/ ¹⁴⁴ Nd	0.51243 ± 4	0.51696 ± 5	0.51247 ± 1	0.51179 ± 2	0.51348 ± 2	0.51175 ± 1	0.51221 ± 1

Clean garnet and clinopyroxene fractions in the size range +60–120 mesh were prepared using a Frantz-type magnetic separator. Mineral separates and rock powders were finely ground in a small agate ball mill, leached for 30 min in 6 M HCl and dissolved in a mixture of HF and HNO₃, in sealed Teflon bombs. Samples were taken up and aliquots collected for spiking in 2.5 M HCl. Rare earth element analysis was done in three fractions using standard isotope dilution techniques²⁰. Nd isotopes were measured as the metal species, normalized to $^{146}\text{Nd}/^{144}\text{Nd} = 0.7219$. Repeated analyses of BCR-1 yielded $^{143}\text{Nd}/^{144}\text{Nd} = 0.51262 \pm 2$. Rock analyses were by X-ray fluorescence spectroscopy²¹ on previously ignited samples. Mineral analyses were by Cambridge Geoscan²². Fe in garnet was taken as FeO. Fe²⁺/Fe³⁺ ratios in pyroxene were obtained by Mössbauer spectroscopy at Trinity College, Dublin. ND, not determined.

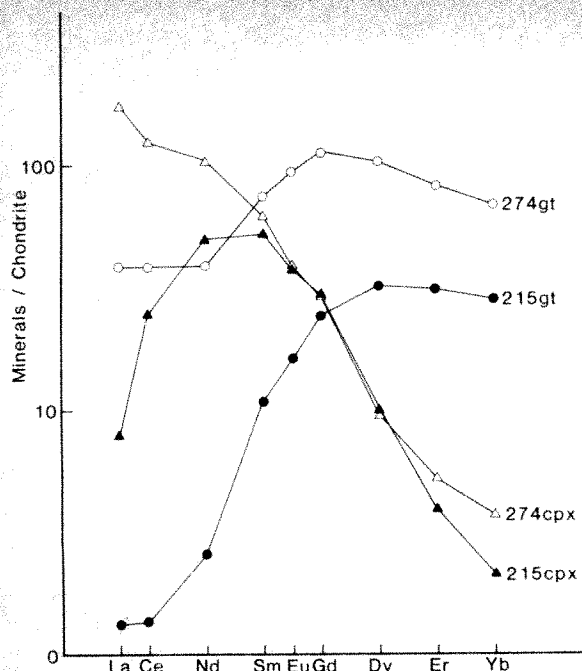


Fig. 2 Rare earth element variations in coexisting garnet-clinopyroxene pairs.

rapid. We infer, therefore, that metamorphism occurred at ~1,100 Myr, that is, during the Grenville orogeny. Of course, it is possible that the eclogite metamorphism was even earlier, and that Grenville overprinting caused thermal resetting of the isotopes without otherwise affecting the mineral compositions. However, this seems unlikely because retrogressive metamorphism observed at Glenelg is, at most, of lower amphibolite facies (<600 °C) and Sm-Nd mineral isochrons are known to survive such metamorphic conditions⁷. The isochrons presented here have certainly survived Caledonian reworking.

Also, consistent with the estimated blocking temperature, equilibrium distribution of rare earth elements seems not to have occurred in eclogites formed below ~600 °C (ref. 8). Where, as in the present case (Fig. 2), equilibrium was achieved, the distribution coefficient $K_D^{\text{gt-cpx}} (= \text{Sm}/\text{Nd}_{\text{gt}}/\text{Sm}/\text{Nd}_{\text{cpx}})$, where gt and cpx represent garnet and clinopyroxene respectively) will presumably vary inversely with temperature. The Glenelg eclogites ($K_D = 3.6-4.1$) therefore imply a higher closure temperature than the Scourie granulites ($K_D = 4.6-5.8$) even though the latter record higher metamorphic temperatures. The higher closure temperature at Glenelg is thought to reflect more rapid cooling.

A Grenville age for the eclogite is significant because it implies that the eastern Glenelg Lewisian lies east of the Grenville front. The age supports the suggestion that the basement to the Caledonian belt in Scotland underwent Grenville reworking⁹, and it leads us to speculate that the Moine thrust is a reactivation of the older Grenville front. The precise location of the Grenville front in the vicinity of Loch Duich appears to be the boundary between the eastern and western Glenelg Lewisian, as the latter preserves hornblende and biotite K-Ar ages of 1,600-2,200 Myr¹⁰.

What was the tectonic framework for generating eclogite so close to the Grenville front? The compositional data in Table 1 permit a general estimate of metamorphic temperatures and pressures to be made. Garnet-clinopyroxene geothermometry suggests a temperature close to 700 °C (ref. 11), at which a pressure >12 kbar would be required to stabilize eclogite of tholeiitic composition¹². Textures in associated eclogite facies feldspathic gneisses indicate intense, syn-metamorphic shearing on planes now dipping gently to the east⁴. The observed combination of pressure, temperature and strain suggests crustal imbrication, the Grenville crustal segment possibly having been thrust westward over the Hebridean craton.

What are the implications of Grenville eclogite in the basement for the depositional age of the unconformably overlying Moine metasediments? In marked contrast to the conditions for eclogite facies recorded in the basement, the Moine cover along the western margin of the Caledonian orogenic belt shows no sign of having exceeded conditions for lower amphibolite facies¹³. Also the local Moine stratigraphy straddles the boundary between the eastern and western Lewisian¹⁴. We therefore favour the view that Moine sediments were deposited on a deeply eroded Grenville basement. Viewed in this light, the Rb-Sr whole-rock isochrons of ~1,000 Myr for the southwestern Moines¹⁵⁻¹⁷ would be interpreted as dating post-tectonic sedimentation and diagenesis rather than regional metamorphism. The ⁸⁷Sr/⁸⁶Sr initial ratio, which is uniform at 0.709 over a wide area¹⁷, may reflect the average value for the source of the Moine sediments. Incidentally, the ~1,000 Myr isochron from the Torridonian Stoer Group has the same initial ratio¹⁸.

Returning to the eclogites, the Nd model age for the whole rock sample with a low Sm/Nd ratio (S274) is ~1,700 Myr. This is the time at which the igneous precursor to the eclogite would have formed from mantle material of chondritic Sm/Nd and ¹⁴³Nd/¹⁴⁴Nd ratios¹⁹. The age is consistent with the basement east of the Moine thrust having experienced considerable mid-Proterozoic magmatism⁹. However, it would be unwise to attach too much significance to this model age as the other whole-rock sample analysed (S215), together with a third (S135 from grid ref. NG811164, Table 1), yielded unrealistic Nd model ages. Both have near-chondritic Sm/Nd ratios, but low ¹⁴³Nd/¹⁴⁴Nd ratios, which may reflect partial isotopic exchange with 'old' Nd in the associated Lewisian metasediments, possibly at the peak of eclogite metamorphism.

Received 5 June; accepted 21 September 1984.

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Tocopherols as likely precursors of pristane in ancient sediments and crude oils

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Pristane and phytane, C₁₄ and C₂₀ isoprenoid alkanes respectively are major components of many ancient sediments and crude oil. The phytol side chain of the chlorophyll a molecule was believed to have been the natural source of these hydrocarbons, but the mechanism by which phytane and pristane are generated from the phytol moiety of chlorophyll, under geological conditions, remain

nuclear. Here we suggest that tocopherols, also known as E vitamins, are likely sources of pristane found in ancient sediments and crude oils, as both flash pyrolysis and thermal degradation of α -tocopherol yield prist-1-ene as a major product. The organic moieties in the kerogens from which prist-1-ene is generated were previously shown to be similar to those yielding pristane under natural conditions¹. As a consequence, pristane and phytane might very well originate from different precursors.

Fifty years ago, Alfred Treibs² demonstrated the presence of porphyrins in ancient sediments and crude oils. These compounds indicated the biological origin of petroleum: their common tetrapyrrolic skeleton suggested that they were derived from chlorophylls biosynthesised in photosynthetic organisms. Thus, the abundance of phytane and pristane found in extracts of ancient sediments and crude oils in the early days of gas chromatography^{3,4}, was quite reasonably thought to have come from the major chlorophylls *a* and *b*, which have a phytol side chain with an isoprenoid carbon skeleton similar to that of phytane and pristane.

The abundance of these isoprenoid hydrocarbons in geological materials has stimulated organic geochemists to investigate possible diagenetic pathways by which these compounds can be formed from phytol. Simulation experiments with phytol^{5,6}, and also *in situ* incubation experiments with ¹⁴C-phytol^{6,7}, have established the conversion of phytol to other functionalised isoprenoids in recent sediments. Unsaturated isoprenoid hydrocarbons such as phytene, phytadienes and phytol dimers and trimers are main products when phytol is heated to 60 °C under different conditions⁶. In thermodegradation experiments with intact sediments at relatively high temperatures (100–300 °C)^{8,9}, phytane, pristane and other isoprenoids are produced; these are all considered to be degradation products of the phytol side chain of the chlorophyll molecule. It is questionable, however, whether such thermodegradation studies accurately simulate natural diagenetic processes. During the past twenty years other isoprenoid compounds have been found in organisms and may also be considered as direct or ultimate sources of acyclic isoprenoid hydrocarbons in sediment samples—for example, phytylesters in aquatic mosses¹⁰, pristane and pristenes in zooplankton¹¹ and diphytanylethers in archaebacteria^{12,13}.

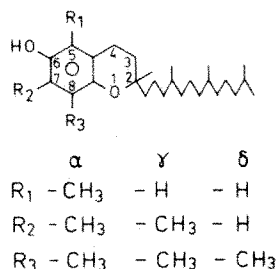


Fig. 1 Structure of tocopherols.

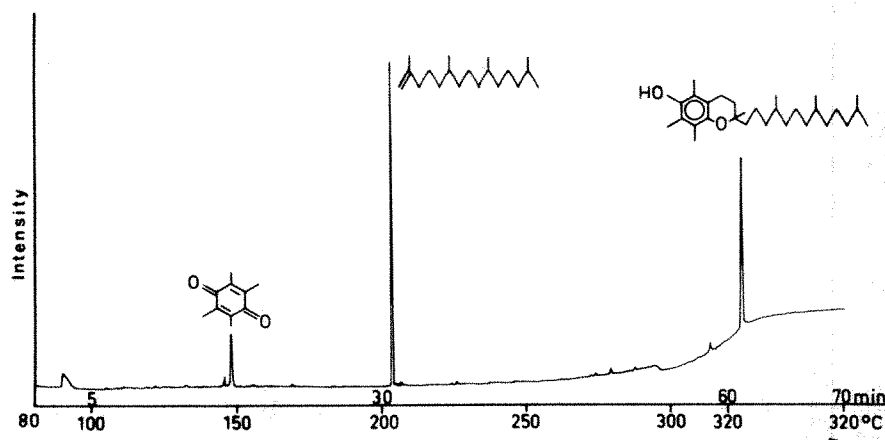
Flash pyrolysis studies of many immature sediments and kerogens have demonstrated that prist-1-ene is a major and sometimes dominant product in the pyrolysate^{14,15}. The flash-pyrolysates of certain organisms and the uppermost layers of sediments have also been found to contain small amounts of prist-1-ene^{14,16–18}.

A detailed study of a suite of Paris Basin samples of increasing maturity showed a good correlation between maturity and the concomitant disappearance of the pyrolysis product prist-1-ene and the generation of pristane¹. This phenomenon indicates that a common precursor, present in the kerogen, generates pristane during maturation, and prist-1-ene on flash pyrolysis. The ability of several isoprenoids to generate prist-1-ene on flash pyrolysis has been assessed to find possible biological precursors for pristane. Dihydrophytyl-palmitate¹⁸, chlorophyll *a*¹⁸, diphytanylethers, phytol and dihydrophytol (J. W. de Leeuw *et al.*, unpublished results) were found to yield mainly phytene or phytadienes. Prist-1-ene is either not present or is only a trace component in the pyrolysates, except for that from palmitylphytanate¹⁸. Although esterified phytanic acid is present in very recent sediments^{19,20}, it is either absent or present in trace amounts in sediments beyond the early stages of diagenesis. Thus, the isoprenoid compounds investigated by flash pyrolysis are not considered to be major sources of pristane in ancient sediments and crude oils. In addition, the type of structure which generates prist-1-ene on flash pyrolysis differs from that which yields straight chain hydrocarbons as these occur as a series of alk-1-enes and alkanes, whereas pristane is absent even when prist-1-ene is the most abundant component in the pyrolysates¹⁴.

Recently, tocopherols (see Fig. 1) have been found in a wide variety of sediment extracts^{21,22}. Their common occurrence in sediments suggests that these compounds—either free or bound—may have an important role in the genesis of isoprenoid hydrocarbons. Therefore, α -tocopherol was pyrolysed under conditions routinely used to investigate kerogens by Curie-point pyrolysis–gas chromatography–mass spectrometry¹⁴. Two major pyrolysis products were encountered: 2,3,5,6-tetramethyl-2,5-cyclohexadiene-1,4-dione (duroquinone) and prist-1-ene (see Fig. 2). The compounds were identified by comparison of their retention times and mass spectra with those of reference compounds.

A thermal degradation experiment with α -tocopherol was also undertaken (N₂; 350 °C; 6 h) and the results were compared with those obtained by Fernholz in 1937²³. Our results support those of Fernholz to some extent: prist-1-ene and duroquinone were major products here, whereas the other components present such as C₁₃, C₁₆ and C₁₈-isoprenoid alkanes, were probably secondary products of prist-1-ene. Figure 3 shows the mechanism proposed for both the flash pyrolysis and thermal degradation. Because tocopherols are highly reactive compounds²⁴, which can easily react with each other and with other naturally occurring compounds, it is thought that the majority of tocopherols will be incorporated into a complex macromolecular

Fig. 2 Pyrolysis gas chromatogram of α -tocopherol. Conditions: Curie point pyrolysis at 610 °C; gas chromatographic separation on a fused silica column (length = 25 m; $\phi_i = 0.31$ mm) coated with CPSil 5 (Chrompack); temperature programme: 80–320 °C at 4 °C min⁻¹, held at 320 °C for 15 min; flame ionization detector (FID) detection).



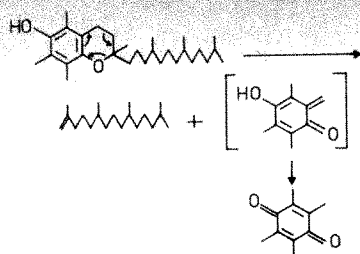


Fig. 3 Proposed degradation mechanism of α -tocopherol.

structure, probably via the phenolic hydroxyl group or the 5-methyl group which is very reactive under oxidative conditions. However, their major pyrolysis product should nevertheless be prist-1-ene formed by the intramolecular rearrangement indicated in Fig. 3. The stereochemistry of pristane in immature sediments²⁵ is also consistent with that of α -tocopherol²⁶.

The flash pyrolysis results obtained for α -tocopherol and the presence of free tocopherols in many sediments^{23,24} suggest that tocopheryl moieties can be major sources of pristane in ancient sediments and crude oils. Tocopherols are relatively abundant in most photosynthetic organisms such as higher plants, algae and cyanobacteria²⁷⁻³¹. Recently, many bacteria have been reported to contain tocopherol derivatives³². Amounts of 10 to >1000-mg kg⁻¹ dry body weight are not exceptional for the concentration of free tocopherols in organisms. These quantities might be underestimates as almost nothing²⁹ is known about the occurrence of non-extractable tocopheryl moieties in biological systems.

The relatively great abundance of chlorophylls in photosynthetic organisms (molar ratio tocopherol/chlorophyll = 0.01-0.12²⁷) does not imply that other compounds, such as tocopherols, can be ruled out as precursors of pristane in the geosphere; the potential survival of a compound during early diagenesis may be more important. Early in diagenesis, phytol is liberated from the tetrapyrrole macrocycle of chlorophylls and is a labile compound. A very high percentage of the phytol biosynthesised by primary producers is probably metabolised within the food web. In tocopherols, by contrast, the isoprenoid moiety is bonded via a carbon-carbon bond to an aromatic nucleus, favouring survival during early (bio)chemical diagenesis. Moreover, tocopherols may escape biodegradation once incorporated into macromolecular structures.

In conclusion, although tocopherols can now be considered as likely precursors for pristane, in view of their pyrolytic behaviour, their relatively high concentrations in organisms and their expected survival by incorporation into kerogen, the generation of phytane from this source is less likely because α -cleavage next to the aromatic moiety is highly unfavourable. As a consequence, pristane and phytane may very well originate wholly or in part from different precursors.

This work was partly supported by the Netherlands Foundation for Earth Science Research (AWON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

Received 11 July; accepted 28 September 1984.

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Accelerator mass spectrometry radiocarbon ages of amino acid extracts from Californian palaeoindian skeletons

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A decade ago, aspartic acid racemization ages were determined for some skeletal remains found in California, near La Jolla, Del Mar and Sunnyvale, suggesting that people were present in North America during the Upper Pleistocene^{1,2}. These ages were obtained from the aspartic acid racemization rate, which was calibrated using a radiocarbon date of $17,150 \pm 1,470$ yr BP determined from a skeleton found in Laguna Beach, California^{3,4}. These studies generated an intense controversy not only about the antiquity of human beings in the New World^{5,6} but also about the validity of racemization-based ages⁶⁻⁹, and prompted efforts to date the finds by other means^{6,7,10,11}. Here we have used accelerator mass spectrometry (AMS) to determine the radiocarbon ages of the amino acid extracts used in the original racemization studies. Our studies indicate that some of the controversial Californian skeletons, which had been assigned to the Upper Pleistocene, are in fact Holocene.

The dating of the first human migrations into the Americas is one of the most controversial topics in prehistory. An accurate time scale for colonization is necessary to assess how rapid hunters and gatherers could expand into virgin territory, and also to evaluate how much time was required for the development of the ethnic and linguistic diversity exhibited by the New World populations. Many archaeologists^{12,13} favour a 'late' entry of ~12,000-13,000 yr BP. However, dates of this magnitude are found even for southern South America¹⁴⁻¹⁶. Therefore it seems necessary to postulate that human groups first crossed Bering much earlier, despite recent reviews of the archaeological evidence which find few convincing sites^{17,18}. Of central importance

Table 1 Radiocarbon and uranium series ages, extent of aspartic acid racemization and level of amino acid preservation in several palaeoindian skeletons from California

Label no.	Sample	Radiocarbon age Oxford	Other	U-series age	D/L-Asp	% Amino acids remaining	Ref.
OxA-152	Stanford	4,830 ± 150	5,130 ± 70		0.14	39	2
OxA-153		4,950 ± 130					
OxA-154	SDM 16709	8,470 ± 140	8,360 ± 75	3,350 ± 800	0.19	4-11	29, 31, 32
OxA-189	Laguna	5,100 ± 500	17,150 ± 1,470 > 14,800		0.25	5	1, 3, 4
OxA-186	La Jolla	5,600 ± 400	1,770 ± 790 1,850 ± 200 1,930 ± 200 5,331 ± 245 6,326 ± 253		0.36		1, 7
OxA-187	Sunnyvale	6,300 ± 400	3,600 ± 600 4,390 ± 150 4,650 ± 400 4,850 ± 400	8,900	0.51	1.4	2, 6, 11
OxA-188	Del Mar	5,400 ± 120		11,800	0.53	2	1, 2, 11

ance in this controversy are the ages of various palaeoindian skeletons found in western North America.

The total amino acids present in the various bone samples (5–10 g) were isolated using procedures described elsewhere^{19,20}. Enantiomeric analyses indicated that the aspartic acid obtained from the La Jolla Shores, Del Mar and Sunnyvale skeletons was extensively racemized. When compared with the extent of aspartic acid racemization (that is, D/L-Asp ratio) measured in the skeleton found near Laguna Beach, which had a conventional radiocarbon date of 17,150 ± 1,470 yr BP, these skeletons appeared to be 30,000–50,000 yr-old^{1,2}. A portion of the original amino acid extracts used in these racemization studies was preserved either frozen or as the dried residue. These extracts, containing 5–10 mg amino acids, together with those isolated from several other skeletons previously dated by radiocarbon, were prepared and analysed by AMS^{16,21,22}. Our AMS-radiocarbon ages and the measured D/L-Asp ratios are listed in Table 1, together with the radiocarbon and uranium-series ages obtained by others. The amount of amino acids preserved in the various bone samples (relative to that in modern bones) is also given in Table 1. The AMS-radiocarbon dates indicate that all of the skeletons, including Laguna, are of Holocene age.

The radiocarbon ages determined using the total amino acid extracts isolated from the Stanford (I) and SDM 16709 skeletons agree well with the ages determined from conventional radiocarbon measurements using the HCl-insoluble ('collagen') component. Our ages for the Sunnyvale skeleton are ~2,000 yr older than the radiocarbon ages of other organic fractions isolated from this skeleton, but the results still indicate that it is Holocene. Therefore we find that the ages obtained by analysis of the total amino acids are comparable with those obtained using either the HCl-soluble or the HCl-insoluble organic fractions.

The radiocarbon age obtained for the La Jolla Shores skeleton agrees with a previously determined AMS age¹⁰ of this skeleton, whereas those obtained using the conventional radiocarbon method are much younger²³. This may reflect the presence of bones of various ages on the site, or we must assume that the fractions used in the conventional measurements were extensively contaminated. In contrast, the AMS-radiocarbon age of amino acids isolated from the Laguna Beach Skeleton is much younger than that obtained, almost 15 years ago, by conventional beta-counting using the HCl-insoluble, gelatinized fraction^{3,4}. The difference in ages for this skeleton is difficult to account for, and may indicate that the total amino acids isolated were not entirely derived from collagen but also contained secondary amino acids from the surroundings. However, based on the good agreement between our amino-acid based ages and those obtained using other organic components, for several of the other skeletons studied, we conclude that Laguna is more likely to be Holocene than Upper Pleistocene as previously assigned.

If indeed the case, this implies that all the aspartic acid racemization ages derived for other specimens, using an age of 17,150 ± 1,470 yr BP for the Laguna skeleton, are too old. Using the newly-determined 5,100 ± 500 yr age for Laguna yields an aspartic acid racemization rate constant, $k_{asp} = 3.6 \pm 0.4 \times 10^{-5} \text{ yr}^{-1}$, giving ages for Sunnyvale and Del Mar of ~14,000 yr BP. Although this age still is older than the AMS-radiocarbon ages of the skeletons' amino acids, it does demonstrate that both Sunnyvale and Del Mar are not of very great antiquity. The k_{asp} value obtained using the 5,100 yr-old age for Laguna also suggests that the age of Los Angeles Man, previously estimated by the conventional radiocarbon method⁴ to be >23,600 yr BP, is more probably ~8,000 yr-old; thus the Upper Pleistocene age assignment for this skeleton should also be viewed with scepticism.

The results in Table 1 reveal no clear relationship between the radiocarbon ages of the various skeletons and the extent of aspartic acid racemization. For example, the 8,400 yr-old skeleton, SDM 16709, has a low D/L-Asp ratio, whereas in the Del Mar and Sunnyvale skeletons the aspartic acid is much more extensively racemized even though these skeletons are 2,000–3,000 yr younger. These results contrast with those obtained at other sites around the world, where in general the extent of aspartic acid racemization has been found to be directly correlated with the age of fossil bones^{19,20,24,25}.

Besides this lack of correlation there are also great differences in the amounts of amino acids preserved in the bones of the Californian skeletons of similar age. In fact, the samples in which aspartic acid is extensively racemized have the lowest amino acid contents and, indeed, there appears to be a direct relationship between the extent of racemization and the level of preservation of collagen (as judged by the amino acid contents of the bones). Bone collagen undergoes hydrolysis via a series of reactions, beginning with cleavage at labile peptide bonds such as those containing aspartyl and seryl residues^{20,26}. The peptides liberated by this hydrolysis are further degraded via peptide bond hydrolysis and an internal aminolysis reaction which takes place at the N-terminal end of the peptides^{20,27}. If, during the process of collagen hydrolysis, the aspartyl residues end up primarily at the N-terminal positions of the peptide hydrolysis products, this could give rise to rapid racemization rates as amino acids at the N-terminal position in peptides have been found to have racemization rates greatly exceeding those at other positions²⁸.

The skeletons having low amino acid contents were found to be depleted in 4-hydroxyproline and enriched in the acidic amino acids, although the general amino acid compositions of these samples still somewhat resembled that of collagen. This indicates that the collagenous matter in these bones had undergone severe diagenesis, which may have altered the D/L-Asp

ratio²⁹. The factors which have contributed to the extensive collagen hydrolysis in some of the skeletons are not known, but probably include leaching by ground waters and heating effects resulting from either shallow burial depths or cultural burial practices. Also many of these Californian skeletons come from coastal shell middens in which the presence of carbonates in the burial soils may have greatly accelerated the rate of collagen hydrolysis^{30,31}.

The results in Table 1 show that there are also difficulties with the application of the uranium (U-) series method for dating these Californian palaeoindian remains. In the case of SDM 16709, the U-series age is too young, whereas for the Del Mar and Sunnyvale skeletons the corresponding ages are too old. The U-series age appears to depend on the level of amino acids preserved in the bones. The basic assumption in U-series dating^{11,31} is that bones rapidly acquire uranium from the surrounding soils. Initially, this uranium is in the water-soluble U⁶⁺ oxidation state, but the bone organic matter acts to reduce and fix this to water-insoluble U⁴⁺, which then decays to the daughter isotopes Th and Pa. In this model, the U-series age of a bone will tend to be younger than the bone's actual burial age³¹, which is the case for SDM 16709³². Our results suggest that, for bones in which the organic matter has undergone severe decomposition, some of the uranium may be reoxidized and lost from the system resulting in U-series ages which are older than the actual burial age of the bone.

In conclusion, our results indicate that the palaeoindian skeletons from California, originally thought to be Upper Pleistocene, are more probably all of Holocene age. Moreover, the direct radiocarbon dating, by AMS, of the amino acids isolated from bones provides a means for determining accurate racemization rate constants which are essential in the amino acid racemization method for dating bones.

We thank D. Darling, P. M. Masters, R. A. Schroeder, M. J. Humm and A. D. Bowles for assistance with sample preparation. The accelerator facility at Oxford is supported by grants from SERC. The travel funds for this research were provided by a grant from NATO.

Received 25 July; accepted 26 September 1984.

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Aequorin measurements of free calcium in single heart cells

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The performance of the heart depends on the concentrations of free calcium ions in the cytoplasm of the myocytes. However, direct evidence for changes in free Ca concentration in physiological events during response to drugs and in pathogenesis has been difficult to obtain because of technical problems in measuring free Ca at 10^{-7} M in cells with a volume of only a few picolitres¹⁻³. Here we describe measurements made with the Ca-sensitive photoprotein aequorin³⁻⁵ in single ventricular myocytes isolated from rat heart⁶. We have detected signals from resting and contracting cells, and from cells exposed to media of altered ionic composition (raised K, lowered Na), ouabain and metabolic inhibitors. We report that free Ca in metabolically-poisoned myocytes is remarkably stable and that severe injury to the cell occurs before the free Ca concentration rises above $1-3 \times 10^{-7}$ M, hence cell damage seems to be a cause, not a consequence, of a rise in free Ca. The technique used here should help to resolve many uncertainties regarding free Ca in heart function, and should be particularly valuable for investigating the role of free Ca in ischaemic pathogenesis.

Ventricular myocytes were isolated by perfusing rat hearts with collagenase⁶ and suspended in medium containing 1.8 mM Ca. Healthy, rod-shaped cells were injected to ~5% of their volume with aequorin solution from a micropipette having a tip external diameter of $<0.5 \mu\text{m}$. Photons emitted from aequorin in a single myocyte were detected by a sensitive, low-noise photomultiplier (see ref. 5 and Fig. 1 legend for details). Figure 1a shows a resting signal from a single rod-shaped myocyte; the free Ca concentration in this cell was 2.5×10^{-7} M. The mean resting free Ca concentration for 42 cells was $1.8 \pm 0.61 (\pm \text{s.d.}) \times 10^{-7}$ M, comparable with microelectrode measurements in Purkinje cells from several species³ and close to aequorin measurements made in ferret papillary muscle⁷. However, our value is probably an overestimate because of the contribution made to the signal by occasional phasic contractions in some of the cells in the sample. Similarly, it is possible that published resting free Ca measurements have been distorted by these small transients, so we are reluctant to place any significance on differences between published values for resting free Ca concentration. Figure 1b was recorded from a cell that showed phasic contractions (in which a localized contractile zone ripples from one end of the cell to the other in ~1 s) at a frequency of $\sim 10 \text{ min}^{-1}$. When exposed to $10 \mu\text{M}$ adrenaline, some myocytes started beating either regularly ($\sim 1 \text{ s}^{-1}$) or in bursts (Fig. 1c); these patterns are similar to published patterns of action potentials⁸. The peak height ($\sim 230 \text{ counts s}^{-1}$) and duration of the regularly-occurring transients (data not shown) were distorted by the slow response time of the recording apparatus, but if their true time course was as described by Allen and Orchard⁹, the number of counts in each transient, which was recorded faithfully by fast scalar would correspond to a peak free Ca concentration of $\sim 3 \times 10^{-6}$ M, comparable with Allen's value. Some of the transients showed 'hesitations' on their downward slopes; improvement in data acquisition and analysis are needed to reveal these 'after-contractions' and, perhaps, oscillatory phenomena¹⁰. Figure 1c shows that each burst of transients ($\sim 2 \text{ s}^{-1}$) followed a 10-s period of elevated free Ca ($\sim 9 \times 10^{-7}$ M) which seemed to be unaccompanied by a change in membrane potential⁸. Slow action potentials, mediated by a very slow calcium current ($i_{\text{Ca,s}}$ have recently been described in guinea-pig myocytes¹¹. Spontaneous slow free Ca transients from a rat myocyte are shown in Fig. 1d. The peak free Ca concentration was 2.5×10^{-6} M comparable with estimates for fast transients. Spontaneous slow

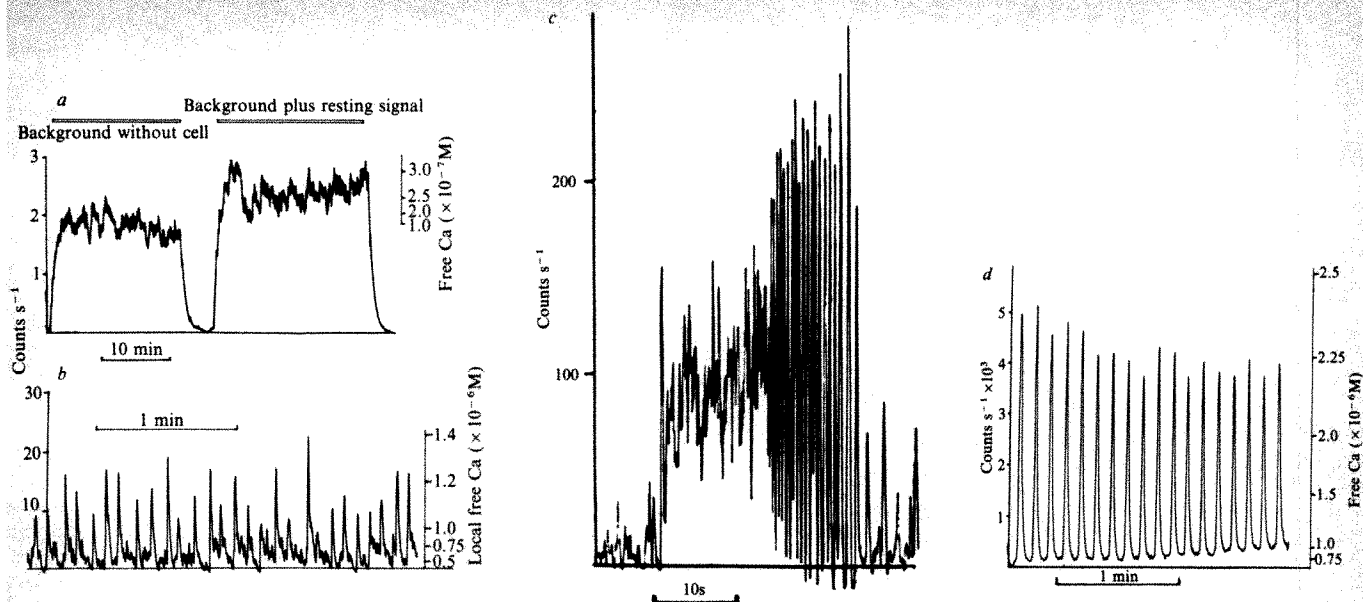


Fig. 1 Aequorin signals from single rat ventricle myocytes, recorded as photoelectron counts s⁻¹. **a**, Resting (diastolic) signal, 0.6 counts s⁻¹ above background (1.8 counts s⁻¹). Ratemeter time constant 33 s. Free Ca concentration was 2.5×10^{-7} M, calculated from the rate of aequorin consumption⁵ knowing the total aequorin counts were 1.8×10^5 . (This recording is from the same experiment as Fig. 2b and corresponds to the first 20 min; note the rod-shaped appearance of the healthy cell.) **b**, Aequorin transients recorded from phasic contractions (ratemeter time constant 1 s). Peak height was ~ 12 counts s⁻¹, total aequorin content 9.4×10^5 counts, hence if the zone of contraction occupied 5% of the cell's volume, the local free Ca concentration would be $\sim 1.2 \times 10^{-6}$ M (see right-hand ordinate). Phasic transients were enhanced in amplitude and frequency in elevated extracellular Ca (3.8, 8 mM), abolished in low calcium medium (0.2 mM), but not detectably changed by either verapamil (10 μ M) or Ni (2 mM). **c**, A burst of spontaneous, fast contraction transients recorded from a myocyte exposed to 10 μ M adrenalin for 3 min before the start of the recording. The peak height and duration of the transients will have been distorted by the relatively slow ratemeter time constant (0.1 s) and by the mechanical inertia of the chart recorder (also responsible for the undershoot below 0 count s⁻¹). The cell contained 1.18×10^6 aequorin counts. **d**, Slow aequorin transients. Peak signals of $\sim 4 \times 10^3$ counts s⁻¹ were recorded faithfully (0.33 s time constant) and corresponded to 2.5×10^{-6} M free Ca (2.57×10^6 counts remained in the cell). Diastolic signals of ~ 200 counts s⁻¹ represented 8×10^{-7} M free Ca. These slow transients started spontaneously 5 min before the start of recording, following a 10-min period when the free Ca concentration rose from 3.5 to 9×10^{-7} M. The amplitude of the transients declined steadily for 6 min after the end of this recording and became unresolvable when diastolic free Ca was 3.2×10^{-6} M. From this criterion, the cell was judged to be in a poor condition.

Methods: The technique was modified from ref. 5 as follows. Aliquots (0.2 μ l) of concentrated aequorin solution (~ 30 mg ml⁻¹) were dialysed for 5 h in a microdialysis tubule against several 100- μ l changes of injection buffer containing (mM): 140 KCl (Aristar) 1 PIPES, 0.025 EGTA, 0.1 EDTA, pH 7.2). Borosilicate micropipettes were filled through the tip by means of a pressure chamber working on the same principle as the air-pressurized equipment described previously but filled with liquid paraffin (mineral oil). Oil pressure of up to 200 atm from an air-to-oil intensifier pump allowed a column of aequorin solution to be forced ~ 0.2 mm into the pipette tip. Myocytes suspended in agarose gel (2%; Sigma IX) within 0.1 mm of the open end of a 3 mm-long flat glass capillary (Camlab microslide) were then impaled with piezoelectric assistance under $\times 600$ magnification and injected with aequorin expelled from the micropipette under 0.3–4 atm air pressure. About 25% of injections were successful, the cells remaining rod-shaped; mean total aequorin content was 6×10^5 counts. Experiments were carried out in HEPES-buffered medium of normal calcium concentration (mM): 120 NaCl, 5.8 KCl, 4.3 NaHCO₃, 1.4 KH₂PO₄, 1.5 MgSO₄, 1.8 CaCl₂, 18.7 HEPES, 14 glucose, pH 7.3 at 36–37 °C.

transients have been found only in deteriorating cells with elevated diastolic free Ca (8×10^{-7} M in this cell). It is interesting that both the slow transients (Fig. 1d) and the bursts of fast transients (Fig. 1c) started when the free Ca concentration had risen to 8 – 9×10^{-7} M; the relationship between free Ca and the initiation of spontaneous transients requires further investigation in view of its possible importance in arrhythmias.

The involvement of free Ca in pathological changes brought about by metabolic inhibition, especially during ischaemia and reperfusion, has long been debated^{12,13}. Recent aequorin measurements in papillary muscle have shown that free Ca does not rise rapidly following anoxia or metabolic inhibition⁹; single myocytes responded similarly to inhibition of both oxidative phosphorylation and glycolysis. Figure 2a shows a typical result: exposure of a myocyte to cyanide (2 mM) together with 2-deoxyglucose (10 mM) had no effect on resting free Ca (2.5×10^{-7} M) for ~ 16 min, at which time the free Ca concentration rose slowly over a further 20 min to 5×10^{-6} M. Similar responses were found with the mitochondrial uncoupler CCP (carbonyl-cyanide phenylhydrazone, 0.5 μ M; Fig. 2b). In this experiment, recording of the signal was interrupted to view the cell under a microscope; shortening of the cell started 6 min after adding CCP and was complete 6 min later, yet the free Ca concentration remained at 3×10^{-7} M. In such experiments it was usual for

free Ca to remain at normal resting levels (~ 1 – 3×10^{-7} M), whereas the myocytes shortened to about one-third of their original length. Low concentrations of ATP (~ 10 – 100 μ M) cause tension development or shortening in permeabilized myocytes in zero free Ca^{14–16}; it is conceivable that a similar mechanism caused shortening of our poisoned cells, assisted by changes in the affinity of troponin-C for calcium at low ATP levels¹⁷. A contribution to shortening could arise from potentiation of tropomyosin-modulated myosin S1-ATPase at Mg-ATP concentrations of ~ 30 μ M¹⁸. On the other hand, Piper *et al.*¹⁹ measured levels of 500–1,000 μ M ATP in anoxic, predominantly rod-shaped myocytes that had shortened, on average, by 40%; this level of ATP appears to be at least 10 times too high for shortening to be explained by any of the above mechanisms.

Blockade of either oxidative phosphorylation or glycolysis alone usually had no effect on free Ca concentration or cell length. Cells that had rounded-up spontaneously in normal medium often showed normal, stable, resting free Ca concentrations. The slow rise in free Ca in rounded-up poisoned myocytes (Fig. 2a, b) occurred a few minutes after the appearance on the cell surface of large hyaline blebs ~ 10 – 30 μ m across, similar to those seen on myocytes after prolonged anoxia^{19–21}, whereas rounded-up cells with stable resting free Ca levels were devoid of these large blebs²².

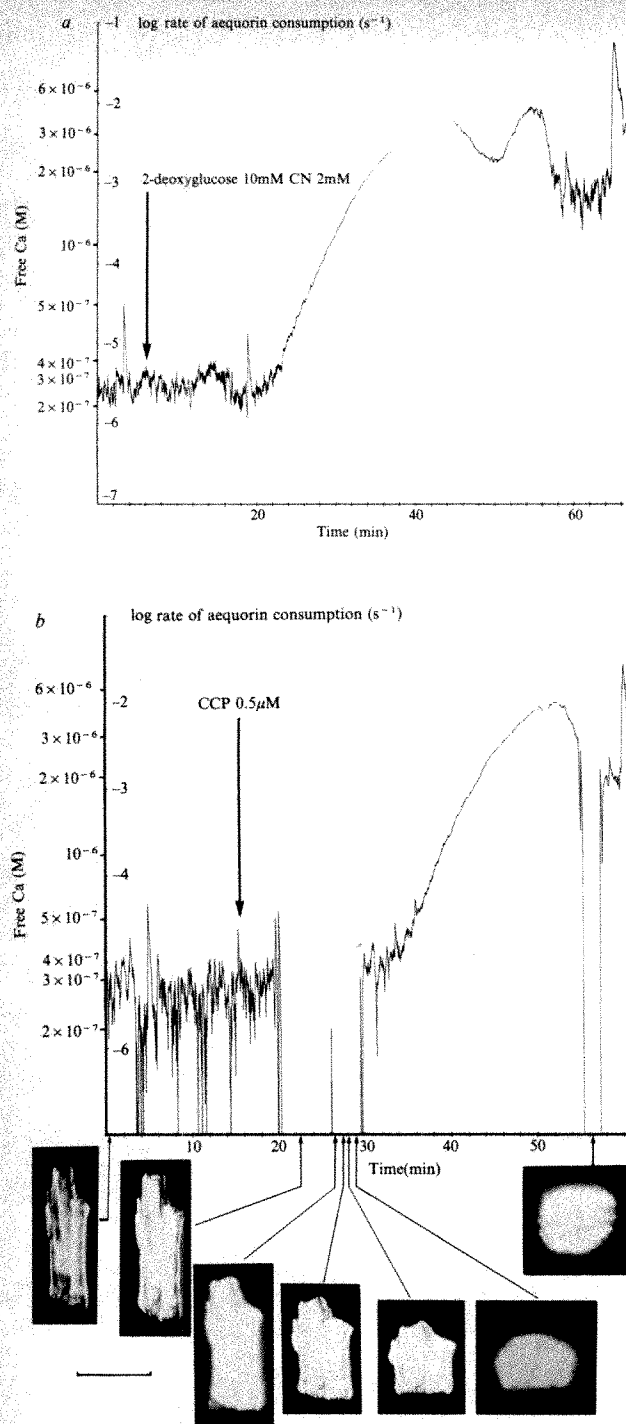


Fig. 2 Effect of metabolic inhibitors on free Ca in single myocytes. A microcomputer-based photon counting system was used to collect data and to plot the log rate of aequorin consumption (s^{-1}) at 1-s intervals. Values of free Ca derived from *in vitro* calibration curves⁵ are also plotted on the ordinate. *a*, Effect of CN (2 mM, fresh) together with 2-deoxyglucose (10 mM, in glucose-free medium). The free Ca concentration remained unchanged at 2.5×10^{-7} M for 16 min then rose over 22 min to 4×10^{-6} M. This is a typical result; the delay between addition of inhibitors and the onset of the rise in free Ca varied from 5 to 45 min. (The plateau at $\sim 5 \times 10^{-6}$ M may be an artefact caused by 'slow-reacting' aequorin; the reappearance of noise in the trace was due to the fall in the count rate as aequorin was consumed, resulting in a rise in statistical noise.) *b*, Effect of the mitochondrial uncoupler CCP (0.5 μ M). The signal recording was interrupted to take photomicrographs of the cell, which show the time course of rounding-up of the cell. The free Ca concentration remained stable during this period at 3×10^{-7} M (confirmed by uninterrupted recordings of other cells, as in *a*). Scale bar, 50 μ m.

We believe that the technique outlined here will help to resolve the relationship between free Ca, metabolic inhibition, altered excitability and structural injury in heart cells. From the preliminary evidence presented here, we conclude that the control of free Ca is not precariously dependent on an abundant supply of metabolic energy and is more resistant to perturbation than is maintenance of normal cell structure.

We thank the British Heart Foundation and the MRC for financial support and Dr K. S. R. Cuthbertson for the use of his prototype computer-based data acquisition system used in the experiments of Fig. 2.

Received 19 April; accepted 10 September 1984.

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Glucose induces closure of single potassium channels in isolated rat pancreatic β -cells

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The major physiological stimulus for the secretion of insulin from the pancreatic β -cell is an increase in the plasma glucose concentration. It is well established that glucose-stimulated insulin secretion is associated with the appearance of electrical activity in the β -cell^{1,2}; glucose concentrations above the threshold level for insulin release produce a slow membrane depolarization followed by either oscillatory bursts of action potentials (5-15 mM glucose) or continuous spiking (>16 mM glucose). Tracer flux studies³ and microelectrode measurements using intact islets of Langerhans⁴ have indicated that the initial depolarization induced by glucose is caused by a decrease in the resting membrane permeability to potassium. Evidence also suggests that the electrical⁵, ionic⁶ and secretory responses^{7,8} to glucose are mediated by the metabolism of the sugar within the β -cell. By using cell-attached membrane patches⁹ from isolated rat pancreatic β -cells we have now identified a potassium channel (G-channel) that is active at the resting potential and is inhibited by glucose. Closure of this channel requires glucose metabolism. This is the first report of a potassium channel whose activity is modulated by glucose and which may couple metabolic and ionic events involved in the secretion of insulin.

The β -cells were obtained from rat islets of Langerhans (see Table 1) and maintained in short-term tissue culture. For single

channel recording, cells were allowed to attach to glass coverslips overnight; over 95% of attached cells stained positively for insulin using an immunoperoxidase method and anti-insulin antibody¹⁰, indicating that most were β -cells. The insulin content of the dispersed cells, determined by radioimmunoassay¹¹, was 3.7 mU per 10^4 cells, comparable to that reported previously¹². We also tested the secretory response of the isolated β -cells by measuring insulin release in response to glucose stimulation (Table 1). Insulin release was increased 2.4-fold by increasing the glucose concentration from 2 mM to 20 mM, and reduced to the basal level in the presence of mannoheptulose (20 mM), which inhibits β -cell metabolism of glucose at the level of hexose phosphorylation^{7,8}. The magnitude of the response to glucose is comparable to that reported previously for dispersed islet cells¹².

To investigate the effects of glucose on the resting membrane permeability to potassium, we recorded single-channel currents from cell-attached patches⁹ using patch pipettes filled with a solution containing 140 mM potassium. In glucose-free solution, the channel observed most frequently at the resting potential had a unit amplitude of approximately -3.5 pA (Fig. 1a). The single-channel current decreased as the membrane was depolarized and reversed at a pipette potential (V_p) of -74.8 ± 1.0 mV ($n = 16$). Figure 1b gives the current-voltage relation of the G-channel, which shows inward rectification. The single-channel conductance, measured between a pipette potential of +80 and -60 mV, had a mean value of 50.6 ± 0.9 pS ($n = 16$). Over the same potential range, the opening probability of the channel showed no significant voltage-dependence. Figure 1a also shows that the G-channel has complex kinetics, with episodes of long closures lasting for several hundreds of milliseconds and episodes of frequent rapid closures of only a few tens of milliseconds. This suggests that the channel has at least two closed states.

Using the whole-cell recording configuration⁹, we determined the β -cell resting potential to be -67.6 ± 2.8 mV ($n = 8$) in glucose-free solution, in agreement with microelectrode studies on intact islets^{2,4}. This value would indicate reversal of the single-channel current at a membrane potential of +7.2 mV, which is close to the estimated K-equilibrium potential of +8.5 mV (taking the internal K concentration to be 100 mM; ref. 4) and suggests that the G-channel is principally permeable to potassium. To confirm this, we carried out three experiments with 70 mM potassium in the pipette (NaCl replacing KCl). In these conditions the extrapolated reversal potential was -58.7 ± 1.3 mV ($n = 3$), corresponding to a 16-mV shift for a 70-mM

Table 1 Insulin secretion by dispersed islet cells

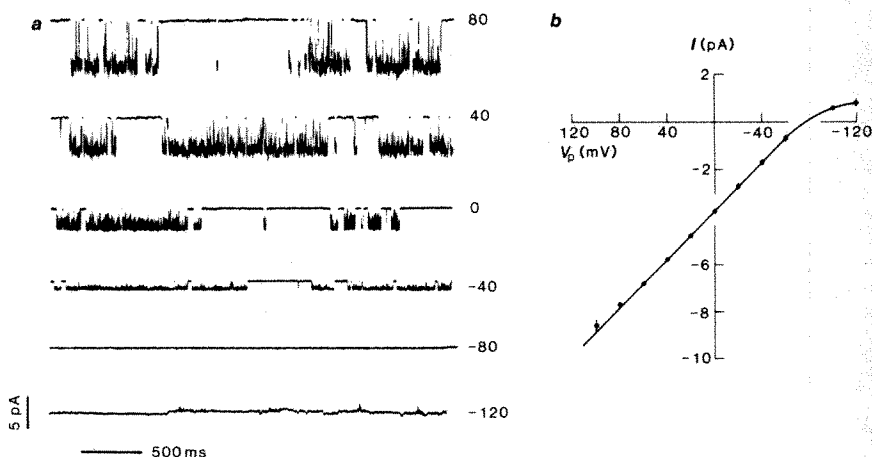
Incubation condition	Insulin release (μ U per 10^4 cells per h)
2 mM glucose	259 $\pm$ 21
10 mM glucose	384 $\pm$ 25 ($P < 0.01$)
20 mM glucose	623 $\pm$ 38 ($P < 0.001$)
20 mM glucose + 20 mM mannoheptulose	268 $\pm$ 19 (NS)

Isolated rat islets of Langerhans were prepared by collagenase digestion¹⁴ and dissociated into single cells by further enzymatic digestion and mechanical dispersion¹⁵. Single cells (2×10^4 cells ml^{-1}) were maintained in RPMI 1640 tissue culture medium supplemented with glucose (11 mM), penicillin (100 U ml^{-1}), streptomycin (0.1 mg ml^{-1}) and fetal calf serum (10%), and incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2 . After overnight culture, cells were collected by centrifugation and washed twice in incubation medium. Incubations (10^4 cells per tube) were then carried out at 37 °C in 0.5 ml HEPES-buffered bicarbonate medium¹⁶ containing 2 mg ml^{-1} bovine serum albumin and the additions stated. After incubation for 1 h, cells were removed by centrifugation and insulin content of the medium was determined by radioimmunoassay¹¹. Values are mean $\pm$ s.e.m. ($n = 10$). Significance of the differences (P), relative to 2 mM glucose, was assessed by a two-tailed Student's t -test. NS, not significant.

change in external K concentration, close to that predicted by the Nernst equation (14 mV) for a K-selective channel. The single-channel conductance decreased to 36.5 ± 0.9 pS ($n = 3$) with 70 mM K^+ in the pipette.

The G-channel was found in 34 out of 41 membrane patches at an average frequency of 1.4 channels per patch. For our pipettes, which had resistances between 3 and 5 G Ω , the patch area is $\sim 5 \mu\text{m}^2$ (ref. 13), indicating a density of ~ 1 channel per $3.5 \mu\text{m}^2$ or roughly 150 channels per cell. We also identified two other channels in cell-attached patches, using pipettes filled with 140 mM K^+ solution. One of these channels had a conductance of 16.9 ± 1.1 pS ($n = 10$) and a unit amplitude of -1.2 ± 0.1 pA ($n = 10$) at the resting potential of the cell, and was observed less frequently than the G-channel (11 out of 41 patches). The other channel was usually activated only at pipette potentials positive to +100 mV, had a unit amplitude of $\sim +6$ pA at +120 mV, a conductance of 119.6 ± 7.8 pS ($n = 8$) and an extrapolated reversal potential (pipette potential) of -74.4 ± 2.3 mV ($n = 8$). The frequency, conductance and voltage-dependence of these two channels suggest that they do not contribute a significant amount of conductance at the resting potential:

Fig. 1 a, Single-channel currents recorded from a cell-attached patch on an isolated rat pancreatic β -cell in glucose-free solution. The potential applied to the inside of the patch pipette (pipette potential) is given to the right of each trace; 0 mV corresponds to the resting potential of the cell (~ -70 mV) and positive pipette potentials correspond to membrane hyperpolarization. Inward currents across the membrane are shown as downward deflections of the current trace and channel openings are inward when the pipette potential is positive to -80 mV and are outward at -120 mV. Experiments were carried out on single cells (10-15 μm in diameter) prepared as described in Table 1, within 1-6 days after plating onto glass coverslips. Similar results were obtained when antibiotics were omitted from the culture medium. The patch pipette was filled with the following solution (in mM): 140 KCl, 5 CaCl_2 , 5 MgCl_2 and 5 HEPES titrated to pH 7.4 with NaOH. The bath solution contained (in mM): 135 NaCl, 5 KCl, 5 CaCl_2 , 5 MgCl_2 and 5 HEPES titrated to pH 7.4 with NaOH. The patch pipettes and recording system were similar to those described by Hamill *et al.*⁹. All experiments were carried out at room temperature (21-25 °C). No corrections have been made for liquid junction potentials¹⁷. Seal resistance = 25 G Ω . Current records were filtered at 0.2 kHz. **b**, Mean relationship between the single-channel current (I) and the pipette potential (V_p) ($n = 16$). Bath and pipette solutions were as described for **a**. The reversal potential corresponds to a pipette potential of -76 mV, and a pipette potential of 0 mV corresponds to the resting potential of the cell. The conductance, measured from the slope of the line between +60 mV and -60 mV, was 51 pS. The line through the points was drawn by eye. Vertical lines indicate $\pm$ s.e.m. where this is larger than the symbols.



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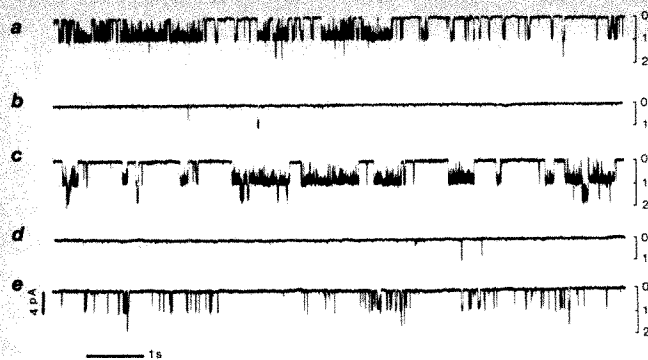


Fig. 2 Effect of glucose on G-channel activity. Single-channel currents recorded at a pipette potential of 0 mV: *a*, in glucose-free solution; *b*, 7 min after changing to a solution containing 20 mM glucose; *c*, 12 min after return to glucose-free solution; *d*, 4 min after perfusion with 20 mM glucose; *e*, 2 min after perfusion with a solution containing 20 mM glucose plus 20 mM mannoheptulose. Records were obtained successively from the same cell. This patch contained two channels and the scale on the right indicates the current level when one or both channels were open. After recovery of activity at the end of the experiment, we confirmed that we were in the cell-attached recording configuration by applying further suction and obtaining the whole-cell configuration⁹. The bath had a volume of 1 ml and was perfused at a rate of ~ 0.8 ml min⁻¹. A slow rate of perfusion was used to avoid rupture of the seal. Analysis of the perfusate indicated that the glucose concentration stabilized within 5 min. Temperature 25 °C. Currents filtered at 0.2 kHz. Seal resistance 25 G Ω .

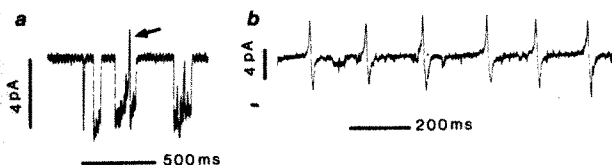


Fig. 3 *a*, Action potential (arrowed) elicited by the opening of a single G-channel in 10 mM glucose solution (12 min). Current waveforms associated with action potentials in the rest of the cell membrane may be recorded extracellularly from cell-attached membrane patches when the input resistance of the cell is high¹⁷. Note that the single-channel currents also decay in amplitude, indicative of a high input resistance. *b*, Example of spontaneous action potentials elicited in response to 20 mM glucose, recorded extracellularly from a cell-attached patch ~ 2 min after changing to glucose solution. In 20 mM glucose, extracellularly recorded action potentials usually decreased in amplitude and become difficult to measure with time. Records filtered at 0.2 kHz.

rather, our results indicate that the resting potential is principally determined by the G-channel.

Figure 2 shows the modulatory action of glucose on the G-channel. Application of 20 mM glucose to the bath resulted in the rapid and reversible inhibition of G-channel activity. In all of six experiments, 20 mM glucose produced a substantial

reduction in channel activity which reached a steady state within 3–10 min and in four of these experiments, glucose removal restored activity over a similar time course. We were unable to test reversal in the other patches because of spontaneous rupture of the seal. Glucose completely abolished G-channel activity in four cases. We were unable to determine the latency of inactivation of the single-channel currents very accurately because a slow perfusion rate was required to avoid rupture of the seal. In the presence of 10 mM glucose, G-channel activity was also substantially reduced (five out of seven patches), three of these showing almost complete inhibition.

Inhibition of channel activity was accompanied by a transient increase in the input resistance of the cell and by the appearance of action potentials. The increase in input resistance was shown both by the relaxation of single-channel currents and by the ability of a single-channel opening to trigger an action potential (Fig. 3*a*). Continuous spiking usually occurred in the presence of 20 mM glucose (Fig. 3*b*), as reported previously for intact islets^{2,4,5}. It is unlikely that the disappearance of the G-channel in the presence of glucose results simply from membrane depolarization because the opening probability of the G-channel is not significantly voltage-dependent between +80 and -60 mV (V_p). Further, channel activity was not restored in the presence of glucose when the pipette potential was changed by 120 mV in either direction.

It is well established that glucose-induced electrical activity⁵, reduced K permeability⁶ and insulin secretion^{7,8} all require the metabolism of glucose. Therefore, in three experiments, we tested the effect of inhibiting glucose metabolism with 20 mM mannoheptulose. In all cases, perfusion with 20 mM glucose plus 20 mM mannoheptulose restored channel activity previously abolished by glucose (Fig. 3*e*). Insulin release from the same preparation of single cells was also inhibited by this concentration of mannoheptulose (Table 1). This suggests that the effect of glucose on channel activity is a consequence of its metabolism.

Thus, we have shown that glucose can inhibit the activity of a channel which contributes significantly to the resting potassium permeability of the β -cell. As the G-channel is normally open at rest, its closure can account for the decrease in membrane potassium permeability^{3,4} and consequent depolarization^{1,2} produced by glucose. G-channel closure must result from an intracellular action of glucose because the pipette solution did not contain glucose and the patch membrane was isolated from the sugar in the bath by the high resistance of the glass-membrane seal. Although we cannot exclude the possibility that glucose and mannoheptulose compete directly for a common receptor site on the G-channel, there is considerable evidence^{6,7} that the effects of glucose and mannoheptulose on both resting potassium permeability and insulin secretion have a metabolic basis. Our results are fully consistent with the hypothesis that channel modulation results from an interaction between some metabolic product of glucose and the G-channel protein(s).

We thank Dr A. Clark for carrying out the immunostaining procedures and Dr B. Ribalet for participating in some of the later experiments. F.M.A. also thanks Drs R. Aldrich, D. Corey and others on the 1983 Single Channel Recording Course at Cold Spring Harbor for instruction in patch-clamp techniques, and Dr M. C. Brown for tissue culture facilities. We thank the MRC and the British Diabetic Association (BDA) for financial support. D.E.H. is a Lawrence Fellow of the BDA.

Received 31 August; accepted 17 October 1984.

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T-cell control of Epstein-Barr virus-infected B cells is lost during *P. falciparum* malaria

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Endemic Burkitt's lymphoma, a tumour of children in which B lymphocytes are infected with Epstein-Barr virus (EBV), is common in areas of Africa where malaria is holoendemic¹. The tumour is characterized by chromosome translocations²; usually the terminal portion of chromosome 8 containing the *c-myc* gene is translocated to chromosome 14³, near the enhancer of the immunoglobulin heavy-chain locus⁴. Less frequent are translocations of chromosome 8 to the κ light-chain locus of chromosome 2 or to the λ light-chain locus of chromosome 22⁵. *In vitro*, EBV induces B cells to proliferate and secrete immunoglobulin and antibody⁶. However, *in vivo* the infected B lymphocytes are under immunological control, so that abnormal proliferation is found only in immunosuppressed patients^{7,8}. Such patients are subsequently liable to develop lymphomas^{7,9}. Burkitt believed that the tumour he had described resulted from interaction between a virus(es) and a "reticuloendothelial system altered by chronic and heavy infection by malarial or other parasites"¹⁰. We report here that during an attack of *Plasmodium falciparum* malaria, T-cell subpopulations are radically altered so that, *in vitro*, B lymphocytes infected with EBV proliferate abnormally to secrete large amounts of immunoglobulin and antibody. This phenomenon offers some explanation for the increased incidence of Burkitt's tumour and the high levels of immunoglobulin found in people living in areas where *P. falciparum* malaria is common¹¹.

Venous blood was taken from patients aged 5–18 yr (mean 9.8 ± 4.7 yr) during and 3 weeks after an acute attack of *P. falciparum* malaria. Parasite densities ranged between 0.5% and 8% (mean 3.1 ± 2.7 %). All patients had antibody titres of at least 1 in 20 to the viral capsid antigen of EBV. Mononuclear cells were separated by buoyant gradient centrifugation on Lymphoprep and used in the regression assay¹² or for determination of lymphocyte subpopulations. Regression assays provide a measure of the strength of T-cell control of EBV-infected B lymphocytes¹². We found that regression indices were high (mean 3.3 ± 1.2) during the attack, denoting loss of control, but that they fell on recovery (mean 1.0 ± 0.4 ; $P < 0.001$, paired *t*-test), signifying a return to normal control (Fig. 1). These 'escaped' B cells produced large amounts of immunoglobulin and a variety of antibodies (Table 1).

It was possible that the autologous plasma in which the regression assays were performed contained a B-cell mitogen¹³ or activator¹⁴, causing proliferation and secretion of immunoglobulin by EBV-infected B cells. To test this idea, we assayed

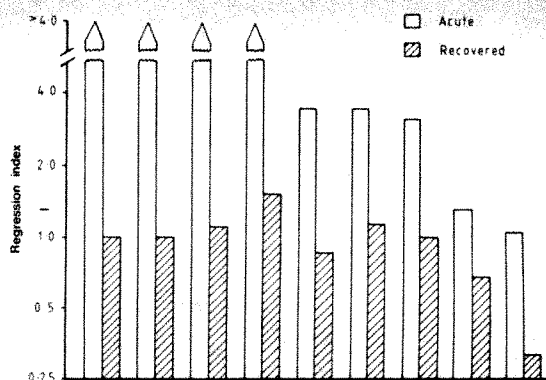


Fig. 1 Regression assays of nine children during the acute and recovered phase of *P. falciparum* infection. The regression index is the minimum number of cells $\times 10^5$ per 0.2 ml microtitre well which are necessary for a 50% frequency of regression of EBV-infected lymphocytes. Mononuclear cells were separated from venous blood on Lymphoprep, infected with EBV at a multiplicity of infection of 0.1 by exposure to B95-8 supernatant for 1 h, washed and then suspended in RPMI-1640/20 mM HCO_3^- with 15% autologous plasma. Doubling dilutions of cells starting at 4×10^5 per 0.2 ml and ending at 0.25×10^5 per 0.2 ml were placed in each of six wells of a flat microtitre plate, incubated for 4 weeks at 37 °C in 5%/CO₂ in air. Half the medium was changed weekly and the cultures were examined for the presence of transformed B cells, characterized by proliferating colonies with hair-like projections, or for regression of these colonies, characterized by dense packing of colonies and then disintegration¹². The 50% frequency of regression was calculated by the method of Reed and Muench²⁵. For statistical purposes, an index of >4.0 was assigned an arbitrary value of 4.1. The mean index of eight healthy adult controls, immune to EBV, was 1.14 ± 0.86 .

mononuclear cells of two normal EBV-immune adult donors in media containing 10–15% of the patient's acute- or recovered-phase plasma. The mean regression indices of six paired assays for the first donor were 0.42 ± 0.14 in acute-phase plasma and 0.66 ± 0.16 in recovered plasma; for the second donor, using five different pairs of plasma, the respective means were 2.12 ± 1.25 and 3.27 ± 1.16 . When the assays were performed in fetal calf serum, the regression indices were 0.40 for the first donor and 2.00 for the second donor.

Having confirmed that plasma factors were not responsible for the high regression indices found during the malarial attack, we explored the possibility that T- and B-cell numbers had been altered by the infection. Table 2 shows that during the attack the number of T cells was reduced; this included both the number and percentage of T-helper cells (T_H) and the ratio of helper to suppressor cells (T_H/T_S). The proportion of B cells was increased, as was the ratio of B cells to suppressor T cells (B/T_S). These findings may explain the high regression indices, for the index is dependent on the number and power of cytotoxic suppressor T cells in the assay¹². These cells were unlikely to have been fully functional, because suppressor cell activity is

Table 1 Immunoglobulin and antibody production by transformed B lymphocytes in regression assays

Patient	Regression index	Immunoglobulin ($\mu\text{g ml}^{-1}$)		Antibody (end dilution)		Sheep red cells
		G	M	<i>P. falciparum</i> IgG	EBV VCA	
1 Acute	4.0	>25.0	58.0	>100	10	16
1 Recovered	1.1	3.2	46.0	10	0	<4
2 Acute	4.0	>25.0	50.0	$>1,000$	1	32
2 Recovered	1.5	3.2	34.0	10	0	<4

Regression assays were performed as described previously¹² except that media contained 15% fetal calf serum (FCS) instead of plasma. After 4 weeks, supernatants from all wells were pooled and tested. IgG and IgM was measured by an enzyme-linked immunosorbent assay²³. *P. falciparum* antibody by an immunofluorescent method using acetone-fixed parasitized red cells as targets, antibody to EBV viral capsid antigen (VCA) by immunofluorescence using the method of Henle *et al.*²⁴ and antibodies to sheep red cells by direct haemagglutination.

Table 2 Number ($\times 10^3$ per μ l) and percentage of T and B cells in blood of 14 patients with *P. falciparum* malaria

Stage of infection	Total lymphocytes		T lymphocytes		T_H lymphocytes		T_S lymphocytes		B lymphocytes			
	No.	%	No.	%	No.	%	No.	%	T_H/T_S	No.	%	B/ T_S
Acute	1.57 $\pm$ 0.83	19.2 $\pm$ 7.5	1.00 $\pm$ 0.66	62.6 $\pm$ 13.0	0.53 $\pm$ 0.38	31.9 $\pm$ 7.2	0.46 $\pm$ 0.26	30.6 $\pm$ 9.3	1.1 $\pm$ 0.3	0.40 $\pm$ 0.27	24.7 $\pm$ 8.7	0.9 $\pm$ 0.4
	1.09	12.5	0.93	10.7	0.72	7.3	0.25	4.9	0.7	0.21	3.4	0.3
<i>P</i> value	<0.001	<0.001	<0.001	NS	<0.001	<0.001	NS	<0.001	<0.001	NS	<0.001	<0.05

Mononuclear cells were separated on Lymphoprep, washed, suspended at 3×10^6 ml $^{-1}$ in RPMI/20 mM HEPES/20 mM HCO $_3$ containing 15% FCS, placed in 1.5-ml aliquots in 2-cm ^{2} wells of a tissue culture plate (Linbro) and incubated overnight at 37 °C. In the morning, all non-adherent cells were removed by pipetting, washed in cold phosphate-buffered saline (PBS)/1.0% FCS/0.1% azide and aliquots reacted at 4 °C with the following antibodies: fluorescein-conjugated F(ab') $_2$ fragment anti-human immunoglobulin (Cappel) and OKT3, OKT4, OKT8 mouse monoclonal antibodies (Ortho). Cell-bound mouse monoclonal antibodies were detected by staining the cells, after washing, with fluorescein conjugated rabbit anti-mouse IgG (Miles Yeda) which had been preabsorbed with human IgG bound to formalin-fixed *Staphylococcus aureus* cells. A drop of each stained lymphocyte suspension was spread on a glass slide, air dried, fixed in ethanol, washed in PBS and mounted under coverslip in a drop of 90% glycerol/PBS before inspection and counting with an epi-illuminated ultraviolet light microscope. Total lymphocyte counts were made on Giemsa-stained blood smears. T_H and T_S refer respectively to helper and suppressor T lymphocytes. NS, not significant. Paired *t* tests were used to calculate *P* values.

known to be decreased in *P. falciparum* malaria ^{15} , perhaps due to a lack of T_H help.

Our findings are supported by data from elsewhere. In Papua New Guinea, where Burkitt's lymphoma is endemic, residents in areas holoendemic for malaria were found to have impaired EBV-specific T-cell immunity compared with residents in non-malarious areas ^{16} . In Sweden, patients with primary *P. falciparum* infections had a lower proportion of T_H cells and a lower T_H/T_S ratio than normal Swedes ^{17} .

Low T_H/T_S ratios are found in a variety of infections, notably acquired immune deficiency syndrome ^{18} , where a human T-cell lymphotropic virus infects T-helper cells ^{19} , resulting in severe immunodepression and greatly increased spontaneous immunoglobulin production by B cells ^{8} . Could malaria activate a latent virus causing T_H -cell destruction, loss of control of B cells infected with EBV and spontaneous immunoglobulin production? The loss of T_H cells was not due to cytotoxic antibodies generated during malaria, for we (data not shown) and others ^{20} have found minimal killing of lymphocytes by acute-phase malaria serum when incubated with complement at 37 °C.

The fact that B cells transformed by EBV are capable of producing large amounts of *P. falciparum* antibody that inhibits the growth of the parasite ^{21} , as well as specific antibody to the virus and heterophilic antibody, is of interest. Children destined to develop Burkitt's lymphoma have abnormally high titres of antibody to viral capsid antigen ^{22} , which might be a signal that large numbers of their B cells are infected with the virus. During repeated attacks of malaria, as a result of loss of T-cell control, these cells would proliferate, increasing the number of cells in which chromosome translocation, and ultimately malignancy, may occur. Another indirect effect of the disturbances set in train by malaria may be that the latent virus, once out of control, switches on immunoglobulin genes in infected cells, thereby producing large amounts of immunoglobulin and making translocations to chromosomes on which these loci lie more likely and frequent. The end results are high serum immunoglobulin levels and an increased frequency of Burkitt's lymphoma following repeated bouts of immunosuppression by the parasite.

We thank Mr S. Rahman, Mr K. Williams and Miss M. Sanneh for technical help. P.S., H.T. and L.W. are visiting postgraduate students.

Received 21 June; accepted 20 September 1984.

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Mucosal mast cells are functionally active during spontaneous expulsion of intestinal mastode infections in rat

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Infestation of the gastrointestinal tract by parasitic nematodes is invariably associated with mucosal mastocytosis ^{1,2} , which is a thymus-dependent phenomenon in parasitized rats ^{3} , and is adoptively transferable with a T cell-enriched population of thoracic duct lymphocytes ^{4} . When derived by *in vitro* culture, mucosal mast cells (MMC) arise from a bone marrow precursor after stimulation by T cell-derived factors ^{5} . In rats infected with the nematode *Trichinella spiralis*, mucosal mastocytosis is temporally associated with the immune expulsion of the adult worms ^{6} whereas in the case of *Nippostrongylus brasiliensis*, mastocytosis is frequently observed to occur after worm expulsion has been completed $^{7-9}$.

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Consequently, there has been doubt as to whether MMC are active and serve a functional role in the expulsion of rat intestinal nematodes. MMC contain and secrete a neutral proteinase, rat mast cell protease II (RMCP II)¹⁰⁻¹³; detection and assay of secreted RMCP II therefore provides a direct measurement of MMC activity. Here we describe the release of this enzyme into the blood of rats infected with *N. brasiliensis* or *T. spiralis*. Our results show that the systemic secretion of RMCP II coincides with the immune expulsion of these nematodes, demonstrating clearly for the first time that rat MMC are functionally active during the immune elimination of primary nematode infections.

Following oral infection of PVG rats with 1,500 *T. spiralis* muscle larvae, most adult worms were expelled from the intestine between 9 and 12 days after infection (Fig. 1), elimination being complete by day 15. Mucosal mastocytosis commenced on day 6 and was maximal at day 12 (Fig. 1). No RMCP II was detected in sera either from uninfected controls or from rats infected for 3 days. By day 6, however, significant ($P < 0.05$; Mann-Whitney U test) concentrations of this enzyme were present in the serum (Fig. 1) and these reached a peak of $4.3 \pm 0.6 \mu\text{g RMCP II ml}^{-1}$ on day 12 of infection (Fig. 1). Serum levels of RMCP II had declined to $2 \mu\text{g ml}^{-1}$ by day 15 and sera were negative for the enzyme 22 days after infection (Fig. 1).

In a second experiment, adult *N. brasiliensis* worms were expelled 10-12 days after subcutaneous infection of Wistar rats with 3,000 third-stage larvae (Fig. 2). Maximal MMC hyperplasia occurred on day 12 (Fig. 2) but, in contrast with infection by *T. spiralis*, this was preceded, on day 6, by a significant ($P < 0.001$, Student's *t*-test) depletion of MMC associated with a significant ($P < 0.001$, Student's *t*-test) reduction of the concentration of RMCP II in jejunal tissues (Fig. 2). These results agree with previous studies^{12,14} and may reflect the release from adult worms of a mast cell degranulating agent¹⁵. The MMC present 9, 10 and 11 days after infection were predominantly immature^{16,17} and their numbers increased during this period, as did the concentration of RMCP II in the jejunal mucosa (for day 6 versus day 9, $P < 0.001$; Student's *t*-test) (Fig. 2). However, whereas mucosal mastocytosis was maximal on day 12, maximal accumulation of RMCP II was not observed in the jejunal mucosa until day 13 (Fig. 2). No RMCP II was detected in sera from either uninfected controls or rats infected for 6 days (Fig. 2), but between days 9 and 11 approximately $0.3 \mu\text{g RMCP II per ml}$ of serum was detected, and the concentration rose to $0.5 \mu\text{g ml}^{-1}$ on days 12 and 13 (Fig. 2).

Because, in the preceding experiment, the serum levels of RMCP II were still elevated 13 days after challenge when most worms had been expelled, a further group of rats was infected with 5,000 *N. brasiliensis* larvae, and the systemic secretion of protease was measured daily between days 6 and 16 after infection. Again, no RMCP II was detected in sera obtained before infection (day 0), but on this occasion there were trace amounts ($119 \text{ ng per ml serum}$) on day 6, and the peak of the response ($1.1 \mu\text{g per ml serum}$) occurred on day 10 (Fig. 3). Between 12 and 16 days after infection, the concentration of RMCP II in the serum fluctuated around 200 ng ml^{-1} (Fig. 3). The immune response, as judged by faecal egg counts, became effective on day 9 of infection and egg counts fell to zero on day 11, which is consistent with major worm expulsion occurring between days 10 and 12, and compared with worm counts on day 9, there was a reduced worm burden on day 10 and parasite expulsion was complete by day 16 (Table 1). The patterns of accumulation of immature MMC and RMCP II in the jejunum (Table 1) were similar to those described in the preceding experiment (Fig. 2).

In marked contrast with the prominent mastocytosis occurring in the jejunum, the numbers of MMC in the ileum increased only gradually (Fig. 2), there being no significant increase between days 6 and 12, the period during which RMCP II was released systemically (Figs 2, 3). The slow rate of mast cell accumulation in the ileum therefore makes it unlikely that MMC in the latter are a major source of secreted enzyme. That the gut is the major source of RMCP II released into the circulation

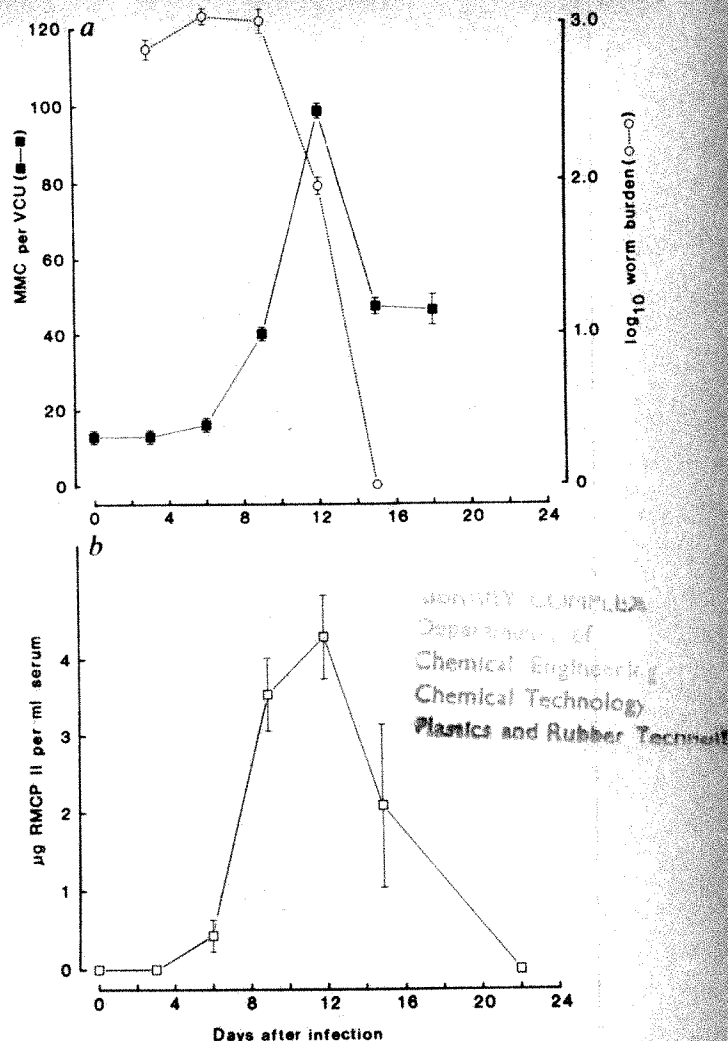


Fig. 1 *a*, Intestinal worm burden (○) and mucosal mast cell counts (■) in the jejunum of rats parasitized with *T. spiralis*. *b*, Concentration of RMCP II (□) in the sera of naive (day 0) and *T. spiralis*-infected rats (vertical bars represent $\pm$ s.e.m.). Twenty-five adult females (8-10 weeks old) were infected orally with 1,500 *T. spiralis* muscle larvae, using standard parasitological techniques³⁰. Rats (3-5 animals per group) were killed at intervals to determine worm burdens and to recover serum samples. Worms were collected by incubating intestines in saline (37°C) using a modified Baermann apparatus³⁰. Samples of jejunum were fixed in Carnoy's fluid and processed for embedding in paraffin wax. Sections (5 μm) were stained with Alcian blue/safranin and MMC were enumerated per villus/crypt unit (VCU)¹²; at least 30 units were counted for each sample. The concentration of RMCP II in serum was assayed by enzyme-linked immunosorbent assay (ELISA)¹³. Serum and tissue samples from four uninfected control rats were also processed and assayed as described above.

Table 1 Mucosal mast cell response to infection with *N. brasiliensis*

Day of infection	Worm burden	ng RMCP II per ml serum	Jejunal RMCP II ($\mu\text{g g}^{-1}$)	MMC per villus/crypt unit
6	4,217 $\pm$ 389	119 $\pm$ 55	16.5 $\pm$ 10.7	0.6 $\pm$ 0.1
9	4,384 $\pm$ 521	956 $\pm$ 175	28 $\pm$ 3	1.4 $\pm$ 0.6
10	3,109 $\pm$ 443*	1,135 $\pm$ 207	30 $\pm$ 4	4.6 $\pm$ 1†
16	0	166 $\pm$ 21	1,755 $\pm$ 256	65.5 $\pm$ 6.0

Details of the experiment are given in Fig. 3 legend. The techniques for quantifying worm burdens, assaying RMCP II in jejunum and serum, and for counting MMC were those described in legends to Figs 1-3. Values are mean $\pm$ s.e.m. * $P < 0.05$; † $P < 0.001$ on day 10 versus day 9 (Student's one-tailed *t*-test).

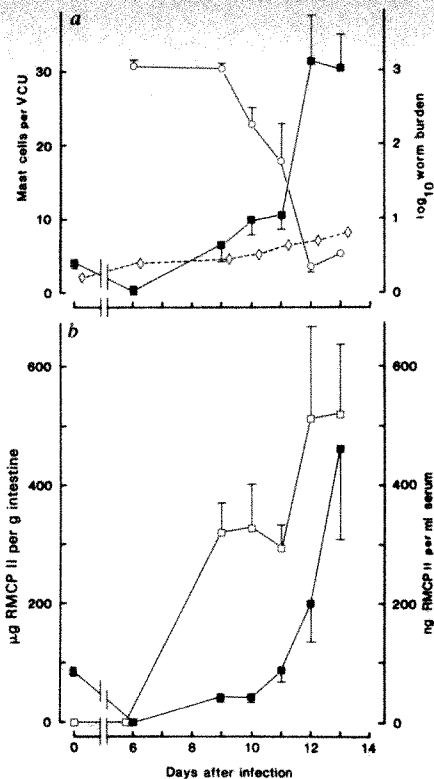


Fig. 2 *a*, Intestinal worm burden (○) and mucosal mast cell counts in the jejunum (■) and ileum (◇) of rats parasitized with *N. brasiliensis*. *b*, Concentration of RMCP II in the serum (□) and jejunum (■) of naive (day 0) and *N. brasiliensis*-infected rats (vertical bars represent $\pm$ s.e.m.). Fifty-seven 6-week-old female Wistar rats were infected subcutaneously with 3,000 *N. brasiliensis* third-stage larvae³¹. Groups comprising 5–6 rats each (four rats only on day 13) were killed at intervals and adult parasites were recovered by allowing the worms to migrate from the intestinal mucosa into saline at 37 °C (ref. 31). Segments of jejunum and terminal ileum were placed in Carnoy's fixative and processed for MMC counts as described in Fig. 1 legend. Lengths (3 cm) of jejunum were examined for the presence of parasites and were washed exhaustively, blotted dry, and weighed before they were homogenized in 3 vol. of 0.15 M KCl. Homogenates were centrifuged (10,000g) and the supernatants assayed for RMCP II by radial immunodiffusion¹². The levels of enzyme in serum were determined by ELISA as described in Fig. 1 legend. Samples from five uninfected control rats (day 0) were processed and assayed in exactly the same way.

was suggested by a recent study which showed that this enzyme is secreted in large amounts into the intestinal lumen during systemic anaphylaxis¹⁸; there was a simultaneous release of RMCP II into the circulation, together with depletion of both MMC and RMCP II from the jejunal mucosa^{13,18}.

The rate of accumulation of RMCP II in the mucosa of *N. brasiliensis*-infected rats lagged behind the development of mastocytosis, in agreement with previous reports which suggested that MMC might secrete rather than store enzyme¹⁹ and monoamines¹⁹. The fact that MMC were immature at the time of maximal enzyme release and that, ultrastructurally, immature MMC have apparent protein synthesizing capacity²⁰, again suggests a secretory function for these cells. Further support for this role is provided by the *in vitro* behaviour of cultured MMC which, during the earliest stages of differentiation, release RMCP II into the culture medium²¹. Immature rat peritoneal mast cells actively secrete histamine²², and it is now clear that *N. brasiliensis* infection in mice generates histamine-producing cell-stimulating factor (HCSF)²³, a lymphokine probably identical to interleukin-3, which recruits putative mast cell precursors to sites of immuno-inflammatory reactions²⁴. Taken together, these studies indicate that immature mast cells and their precursors may be as functionally active as the fully granulated mature

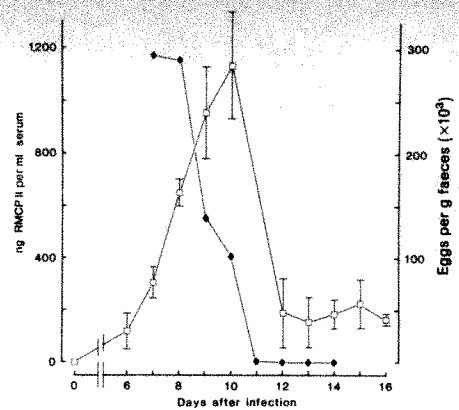


Fig. 3 Faecal egg counts (◆) and concentration of RMCP II in the sera (□) of rats infected with 5,000 *N. brasiliensis* third-stage larvae. The techniques used were similar to those described for Fig. 2 although, in this instance, 22 adult male Wistar rats were infected. A group of five rats was bled via the tail vein on the day of infection (day 0). Groups of 6–8 rats were bled on day 7 and daily thereafter (except day 11), again via the tail vein except those rats exsanguinated on days 6, 9, 10 and 16 after infection. The immune response to the parasite was monitored by faecal egg counts (bulk samples) using standard methods³¹. Concentrations of RMCP II in the sera of control (day 0) and infected rats were assayed by ELISA as described for Fig. 1.

cell, and the present results are compatible with such a possibility.

Our findings indicate for the first time a functional role for MMC during the immune expulsion of intestinal nematodes. Release of MMC mediators may allow translocation, into the gut lumen, of serum antibodies and complement by altering mucosal permeability¹. In addition, these mediators may directly affect the parasites themselves²⁵, may alter epithelial integrity¹⁸ and secretory activity²⁶, and may act on intestinal smooth muscle²⁷. The mechanisms by which MMC are activated, in the absence of detectable parasite-specific IgE^{28,29}, remain to be elucidated.

Received 22 June; accepted 28 September 1984.

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Capacitance measurements reveal stepwise fusion events in degranulating mast cells

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Mast cells undergo an extensive and violent morphological transformation on stimulation¹. Here we describe the dynamics of fusion of the secretory granules in individual mast cells during exocytosis. The cell membrane capacitance (proportional to the cell surface area) was measured using the whole-cell patch-pipette technique^{2,3}, in which the intracellular space is dialysed with the solutions used to fill the patch pipette. Our results show that degranulation occurs spontaneously and reproducibly if the GTP analogue, GTP- γ -S, and Mg-ATP are present in the pipette filling solution. Contrary to previous reports⁴⁻⁸, in these conditions Ca^{2+} (and/or Ca^{2+} buffers) is not required for degranulation. Although electrogenic Ca^{2+} entry was not detected before or during degranulation and membrane conductance remained low, the capacitance, and by implication the area of the membrane of degranulating cells, increased sigmoidally and stepwise. We conclude that stepwise increases of capacitance are due to the fusion of individual secretory granules with the plasma membrane, and that guanine nucleotide regulatory proteins are involved in the control of this process.

To our surprise, especially in view of an earlier report⁹ indicating stimulation of exocytosis following microinjection of Ca^{2+} , perfusion with various combinations of Ca^{2+} and EGTA (to maintain cytosolic free Ca^{2+} in the range 0.2–10 μM) generally failed to induce degranulation in rat mast cells that were otherwise normal. Degranulation of neighbouring cells could be easily induced by adding compound 48/80 in their vicinity, while the dialysed cell remained unaffected.

It has recently been demonstrated that guanine nucleotide regulatory proteins are essential for the transmission of signals controlling many cellular processes¹⁰ and that a GTP-binding protein is involved in the activation of rat mast cells¹¹. When rat mast cells were dialysed through the patch pipette with solutions containing GTP- γ -S at concentrations down to 5 μM , they rapidly degranulated (Fig. 1), even in nominally Ca^{2+} -free solutions. Figure 1a illustrates the appearance of a normal mast cell viewed with Nomarski optics. A glass pipette was pressed and sealed against the cell surface using standard patch-clamp procedures¹². Following a strong suction pulse, the membrane patch enclosed by the pipette ruptured without altering the seal between the glass pipette and the rest of the cell membrane. From this point, the cell was rapidly dialysed—most ions and low molecular weight solutes will equilibrate between the cytoplasm and the effective infinite volume of the pipette within seconds¹³. After a variable lag time, degranulation occurred (Fig. 1b).

The rapid fusion of a large number of secretory granules with the plasma membrane of mast cells produced an increase in the area of the plasma membrane through the formation of large irregular cavities which transformed the morphology of the cell surface (see Fig. 1b). The changes in membrane area were monitored by measuring the cell membrane capacitance (see Fig. 1 legend); a typical protocol for these experiments is illustrated in Fig. 1c. At the start of whole-cell recording (arrow), the capacitance increased markedly (6 pF); this level corresponds to the capacitance of the membrane of the resting cell. After a variable lag time, the cells degranulated, generating a large increase in total membrane capacitance (average 3.9 ± 0.9 -fold, $n = 12$), and reached a new steady state ~3–5 min after the onset of degranulation.

Similar results were obtained when experiments of the type

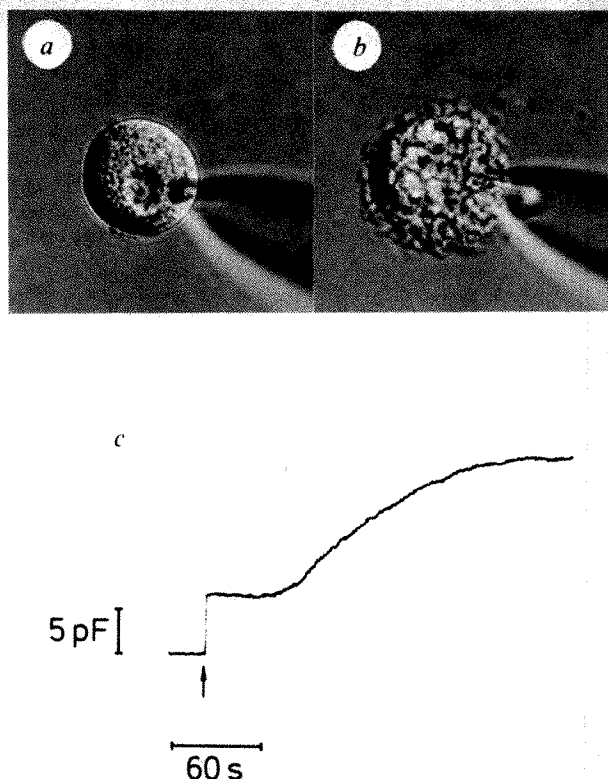


Fig. 1 Effect of dialysis of a single rat peritoneal mast cell with pipette solutions containing GTP- γ -S. **a**, A typical mast cell observed with Nomarski optics on a Zeiss inverted microscope (IM35). The patch pipette can be seen slightly out of focus, coming in at an angle at the lower right-hand corner. This picture was taken after formation of a giga-seal in the cell-attached mode. Typical features of mast cell morphology can be observed at this time, in particular a prominent nucleus and a cytoplasm densely packed with secretory granules. **b**, The same cell photographed ~5 min after the start of dialysis and whole-cell recording. Extensive changes in appearance, corresponding to exocytotic degranulation, can be seen: residues of intragranular matrix material are floating near the cell. The composition of the pipette-filling solution was: 10 μM GTP- γ -S (Boehringer-Mannheim), 100 μM Na-EGTA, 1 mM ATP, 150 mM K-glutamate, 7 mM MgCl_2 , 10 mM HEPES. The external medium comprised (mM): 150 NaCl, 2.8 KCl, 1 MgCl_2 , 6 CaCl_2 , 10 HEPES. All solutions were adjusted to pH 7.2 with NaOH and the osmolality was maintained at 300–320 mosmol kg^{-1} . **c**, A large increase in the membrane area of degranulating mast cells can be measured at the same time as the morphological changes are being observed under the microscope. Changes of area were monitored by measuring the cell membrane capacitance at low frequencies (414 Hz) using the synthetic Pseudo Random Binary Sequence (PRBS) method described by Fernandez *et al.*³. An 18-mV PRBS (128 bits long, 6,621 Hz total bandwidth) was continuously applied to the command input of an EPC-7 patch-clamp (List Electronics). In voltage-clamp conditions (typically 0 mV), the whole-cell membrane current in response to the PRBS stimulus was sampled and stored by a PDP-11/23 minicomputer (Digital Equipment Corporation) operating on-line. The membrane capacitance was then evaluated from the imaginary part of the cell membrane admittance. This method allows accurate determination of the linear parameters of the cell—d.c. capacitance, d.c. conductance and series resistance—over wide ranges as the cell degranulates. The figure shows the time course, before and during degranulation, of the cell membrane capacitance. This was measured in the whole-cell recording mode following identical procedures to those used for **a** and **b** (internal solution contained GTP- γ -S at 50 μM in this case). The arrow indicates the time of transition from cell-attached to whole-cell recording, at which point the contribution of the cell membrane capacitance becomes apparent (typically 5–6 pF). After an initial delay, the cell degranulated and the capacitance increased 3–6-fold. Membrane resistance (not shown) did not change significantly during degranulation. All experiments were carried out at room temperature.

shown in Fig. 1 were repeated using external media in which Ca^{2+} was maintained at submicromolar concentrations (50 μM EGTA and no added Ca). This suggests that extracellular Ca^{2+} is not a requirement for degranulation in the conditions we used. Indeed, the cell membrane conductance remained at a very low level during degranulation and any increases observed (measured in four cases, 180 ± 150 pS) were always very small. This contrasts with the situation pertaining in adrenal chromaffin cells in which exocytosis can be induced by inward Ca^{2+} currents of only ~ 100 pA (in the range 1,000–2,000 pS (ref. 2)).

An estimate for the maximal increment in membrane capacitance can be obtained from stereological electron microscopic observations¹⁴ of the diameter distribution of the secretory granules of rat peritoneal mast cells. According to these data, cells, typically 10.9 μm in diameter, will contain 1,020 secretory granules of ~ 0.77 μm diameter, thus predicting an increase in capacitance from 3.73 pF for the resting cell to 18.96 pF for a cell in which all granules have fused with the plasma membrane. Deviations from this estimate are to be expected because of variation in granule size, and these should be taken into account. Also, in our experiments we have used larger cells (average diameter measured in 15 cells was 12.8 ± 1.4 μm , giving a capacitance of 5.2 pF in the resting state), containing a larger number of secretory granules. Even taking this into consideration, our estimate shows that the largest capacitative changes we have observed can only be explained by postulating that most of the secretory granules fuse with the plasma membrane.

The presence of large secretory granules in rat peritoneal mast cells, and their fusion with the plasma membrane on stimulation, predicts that the increases of capacitance observed in Fig. 1c should occur in a stepwise manner as single granules fuse with the plasma membrane. This is illustrated in Fig. 2 and is consistent with the concept of granule fusion and retrieval as being the mechanism of exo- and endocytosis. Cells degranulate rather slowly at first, after an initial delay (Fig. 1c). When this is observed with high gain, the capacitance is seen to increase in a stepwise manner ('on' steps, Fig. 2a).

We have observed two other types of discrete capacitance changes. Occasionally, particularly when we used filling solutions lacking the GTP analogue and containing high concentrations of EGTA (0.5 mM), the capacitance declined in steps over a period of 5–10 min (Fig. 2b). It is likely that in such situations some degree of degranulation had occurred during the establishment of the whole-cell recording configuration, so that the step decreases of capacitance ('off' steps) represent retrieval of excess membrane. Episodes were also occasionally observed during which the capacitance fluctuated transiently between two levels ('flicker'; Fig. 2c). Although we did not study these two phenomena extensively, we consider that they are probably related to granule fusion and retrieval, as they are not associated with conductance changes of the kind produced by the switching of ionic channels.

Further evidence that the data of Fig. 2 are indeed related to granule fusion and retrieval (exo- and endocytosis) was obtained by comparing the amplitude distribution of these processes. Figure 3a, b shows the amplitude distribution for the on and off steps, respectively. The expanded-scale distributions (Fig. 3a, b, lower panels) show good agreement between the magnitude of most of the capacitance steps and the distributions predicted on the basis of the dimensions of the secretory granules. Note, however, the occurrence of a population of capacitance steps (> 60 fF) which are too large to be accommodated by the fusion or retrieval of individual granules. These larger events are likely to correspond to the fusion or retrieval of multiple fused vesicles, corresponding to what has been described as compound exocytosis¹⁵.

Figure 3c shows the size distribution of capacitance flicker. The large steps are absent from this distribution, and all events fall within the range expected for single vesicle fusion events. Models proposed for exocytosis typically consider vesicle fusion with the plasma membrane to be an irreversible process. In contrast, an attractive way to explain the flicker we have observed

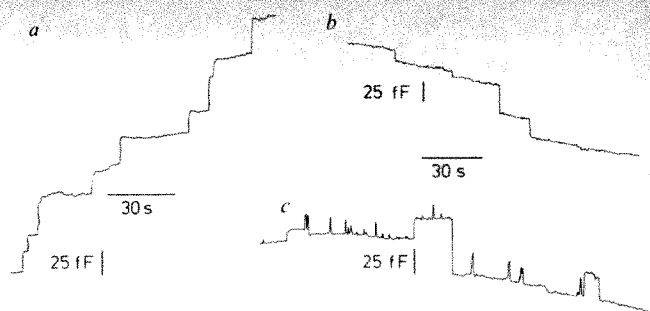


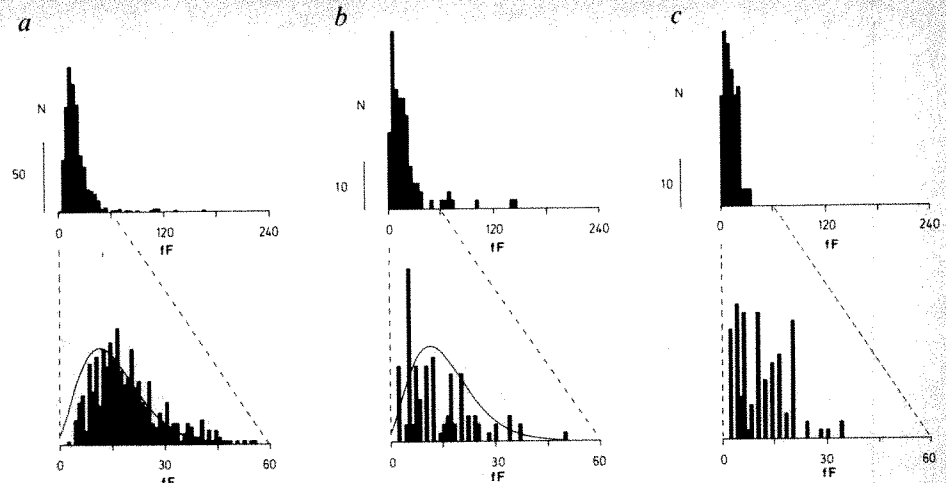
Fig. 2 Stepwise changes in the capacitance of mast cells undergoing exocytotic degranulation. **a**, Typical time course of capacitance changes monitored at the start of degranulation in a rat mast cell dialysed internally with GTP- γ -S. Capacitance increments associated with degranulation occur in a stepwise fashion, in agreement with the concept of vesicle fusion. The step increases ('on' steps) shown are not associated with measurable changes in membrane conductance and occur independently of the membrane potential. Electrode filling solution comprised: 140 mM KCl, 7 mM MgCl_2 , 5 μM GTP- γ -S, 200 μM ATP, 375 μM EGTA, 320 μM CaCl_2 . This particular internal filling solution caused a slow, partial degranulation. (ATP concentrations were kept as low as possible so as to reduce the possibility of ATP leakage from the patch pipettes while they were being brought into contact with mast cells. This would have caused solute permeability lesions to form in the plasma membrane with consequent unwanted effects¹⁷.) **b**, Stepwise decreases of capacitance ('off' steps) measured on cells dialysed with solutions containing no GTP- γ -S and a relatively high Ca^{2+} buffering capacity. In these conditions, the cell capacitance typically falls and this also occurs in a stepwise fashion. Electrode filling solution comprised: 130 mM K-glutamate, 20 mM Na-glutamate, 7 mM MgCl_2 , 10 mM HEPES, 5 mM ATP, 100 μM EGTA, pH 7.2. **c**, Capacitance 'flicker'. Transient increases of capacitance of relatively short duration, usually absent from downward trends like those of **b**. The capacitance flicker can be observed in both the presence and absence of GTP- γ -S, and/or various combinations of Ca^{2+} /EGTA. In the experiment illustrated, EGTA was omitted from the electrode filling solution which was otherwise the same as for **b**. In these experiments, the external buffer solution was identical to that used for the experiments of Fig. 1c except that the concentration of Ca^{2+} was 2 mM.

Methods: Capacitance steps of the kind expected for the fusion of a single secretory vesicle are best measured by the phase-sensitive detection method of Neher and Marty², which is less noisy than the PRBS method used in the Fig. 1c experiment. It cannot, however, track large capacitance changes of the type shown in Fig. 1c, as the method works well only for small deviations from the compensated cell capacitance. In this figure, capacitance was tracked with an 11-mV sinusoidal signal of 800 Hz. Simultaneously, the quadrature component (membrane conductance) was monitored with a second phase-sensitive detector tracking the sinusoidal signal at 90° with respect to the capacitance detector. The conductance as monitored in this way did not seem to be correlated with the capacitance events illustrated, showing only the occasional opening of a single ion channel or nonspecific leakage (see text).

is to assume the formation of a reversible aqueous pore between the secretory vesicle and the plasma membrane, before formation of the (irreversible) fused state. Alternatively, newly fused vesicles might be pinched off by the strong mechanical disturbances that must occur during degranulation, as vesicles fusing with each other and with the plasma membrane are known to cause extensive reorganization of the cell surface¹⁶.

Our results provide a way of monitoring quantitatively the kinetics of granule fusion (and release) in a single cell. A advantage of our approach is the simultaneous dialysis of the cytoplasm with the solutions used to fill the pipette, which enables pipette biochemistry to be carried out concomitantly with the electrical measurements. Our results are surprising in that they show that the introduction of the GTP analogue by dialysis through the patch pipette is a sufficient stimulus to secretion even in conditions where Ca^{2+} is absent both from the

Fig. 3 Size distribution (N) of 'on' (a) and 'off' (b) steps, and capacitance 'flicker' (c). Measurements were collected from 25 different cells in experiments similar to those of Fig. 2a (see Fig. 2 legend for details of conditions). **a**, On step distribution obtained mainly during the earliest degranulation events at the start of secretion and during subsequent phases of slow degranulation in the conditions of Fig. 2a. The expanded scale (lower panel) shows the agreement between the observed step size and that predicted by the vesicle diameter frequency distribution (continuous line) obtained with electron microscopic methods¹⁴. The data for the vesicle diameters were fitted by a normal gaussian distribution having a mean of $0.68 \pm 0.21 \mu\text{m}$ ($\pm$ s.d.). Using these parameters, the capacitance step distribution for fusion of individual granules was predicted as shown and found to agree well with our data. This distribution also predicts that no steps larger than 60 fF should be observed. The large on steps probably correspond to the fusion with the plasma membrane of clusters of previously fused granules (compound exocytosis). **b**, Off step size distribution obtained from non-degranulating cells. The expanded scale (lower panel) once again shows good agreement between the data and the predicted size distribution (same parameters as in a). At small sizes the data deviate significantly from the predicted distribution and this may reflect two types of endocytotic processes occurring simultaneously. **c**, Flicker size distribution. Note the absence of very large sizes as opposed to the data shown in a and b . The expanded scale (lower panel) shows that the flicker size distribution is confined to the limits predicted by single vesicle sizes. We have not attempted to fit these distributions.



pipette and the external solutions. This contrasts with the earlier finding that GTP analogues introduced into mast cells that have been reversibly permeabilized with low concentrations of ATP⁴⁻ causes them to undergo exocytotic secretion of histamine only on subsequent provision of extracellular Ca²⁺ (ref. 11).

The differing requirements for Ca²⁺ in the various experimental systems probably depend on the degree of dialysis achieved. For instance, the conflict can be adequately resolved by postulating that a Ca²⁺-dependent inhibitory protein present in the cytosol of intact cells prevents the direct activation of exocytosis by the GTP analogue. After the loss of this protein through the very efficient dialysis performed with the patch pipette, the analogue might act in a Ca²⁺-independent manner. In intact cells and in cells loaded with GTP analogues following permeabilization with ATP⁴⁻, in which the full complement of soluble proteins is retained, direct action by the nucleotide is prevented by this inhibitory protein. Elevation of intracellular Ca²⁺ would act to remove this inhibition.

Although the site and mode of action of the GTP analogue remains unknown, it seems logical to assume that it binds to a guanine nucleotide regulatory protein, thus activating an as yet unknown chain of events leading to the massive release of histamine and other secreted products. Possible sites for such regulatory proteins are the plasma membrane¹¹, the endoplasmic reticulum and the secretory granule membrane.

The δ - and ϵ -chains of the human T3/T-cell receptor complex are distinct polypeptides

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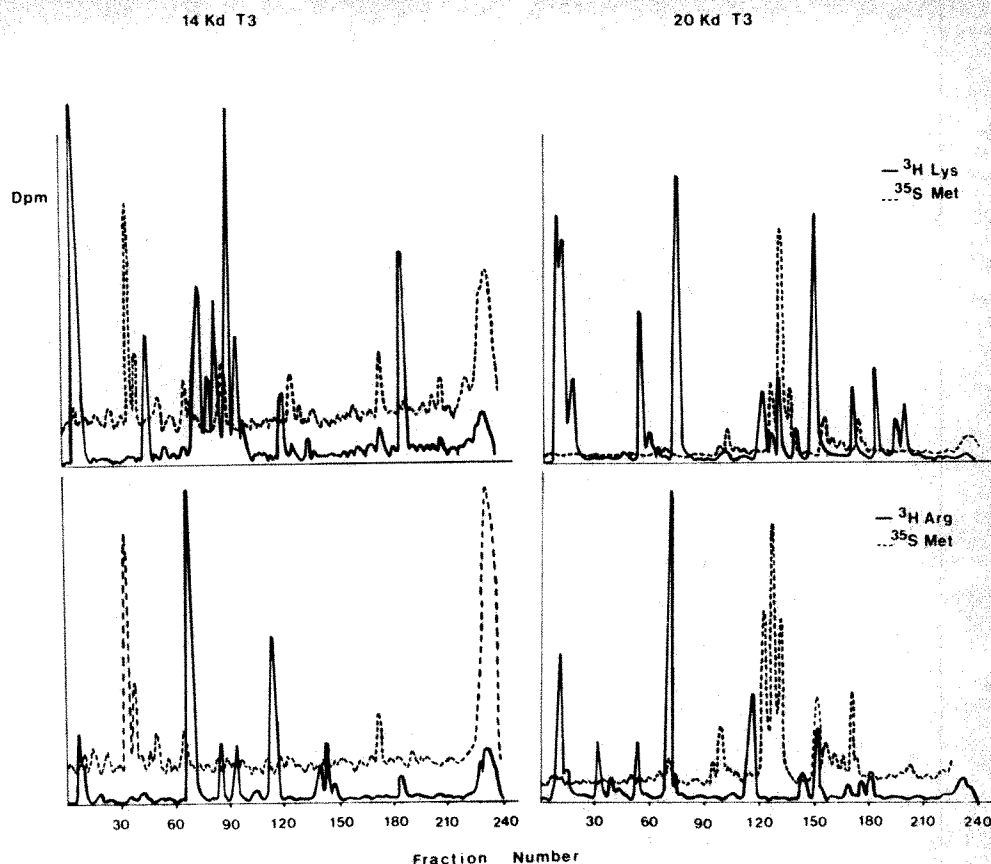
The T3/T-cell receptor complex on the surface of human thymus-derived lymphocytes consists of four glycoproteins: the α -chain of relative molecular mass (M_r) 40,000–50,000 (40–50K), the β -chain (37–45K); the γ -chain (25K) and the δ -chain (20K)^{1–3}. The T3 α - and β -chains have been identified as clonotypic T-cell receptors^{3–7}, but functionally the T3/T-cell receptor chains seem to form a single complex: monoclonal antibodies directed at the 20K T3 components are mitogenic for normal human T lymphocytes^{8,9} and, at higher concentrations, anti-clonotypic and anti-20K reagents block T-cell function^{4,10}. Recently, Zanders *et al.*¹¹ showed that incubation of human T-helper clones with high concentrations of antigen abolishes antigen-specific proliferation and induces disappearance of T3 from the cell surface. Thus, the T3/T-cell receptor complex consists of two variable subunits, the T3 α - and β -chains, which interact with antigen and the monomorphic 20K/25K T3 chains. Recently, the existence of a fifth polypeptide chain, the unglycosylated T3 ϵ -chain, has been postulated^{12,13}. Here we confirm that a 20K ϵ -chain does exist. The T3 ϵ -chain differs from the T3 δ -chain in primary structure as judged by N-terminal amino acid sequencing, peptide mapping and immunoblotting with anti-T3- δ and anti-T3- ϵ antibodies. Treatment with endoglycosidase F revealed two nonglycosylated T3 δ polypeptide backbone chains (16K and 14K) with identical amino termini. Together with previous pulse-chase experiments² this observation suggests that the 14K T3 polypeptide is derived from the 16K T3 precursor by proteolytic processing near the C-terminus of the molecule.

Using differential labelling studies and pulse-chase experiments, we suggested previously that two 20K T3 components might exist¹². We had found that one 20K polypeptide chain

Received 9 July; accepted 17 September 1984.

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Fig. 3 Tryptic peptide analysis of the T3 δ - and T3 ϵ -chains. The T3 δ - and T3 ϵ -chains were isolated from an anti-Leu-4 immunoprecipitate which had been treated as described for Fig. 1a (lane 1). The 14K and 20K material was either metabolically labelled with ^3H -L-lysine and ^{35}S -L-methionine or with ^3H -L-arginine and ^{35}S -L-methionine (see Fig. 1). Reduced and alkylated proteins were eluted from a preparative SDS-PAGE, dialyzed and precipitated with ice-cold acetone in the presence of 100 μg bovine γ -globulin as a carrier. The lyophilized protein was dissolved in H_2O , precipitated with acetone, resuspended in 0.05 M NH_4HCO_3 , pH 7.4, TPCK-trypsin (Millipore) in 0.001 M HCl added at a ratio of 10:1 (carrier protein:enzyme), and incubated at 37 $^\circ\text{C}$ for 4 h. The digest was lyophilized and the tryptic peptides were resolved by reverse-phase HPLC on a Supelcosil C_{18} column (150 $\times$ 4.6 mm i.d., Supelco) at a flow rate of 1 ml min $^{-1}$ with a Spectra-Physics SP8000B liquid chromatograph. After initial isocratic elution for 5 min in 0.1% CF_3COOH , linear gradient elution to 0.1% CF_3COOH /30% CH_3CN was run for 95 min followed by gradient elution to 0.1% CF_3COOH /100% CH_3CN in 10 min. Fractions were collected at 0.5-min intervals and analysed by liquid scintillation counting.



ponents were isolated from large amounts of HPB-ALL plasma membrane fractions (see Fig. 1) by elution from immunoabsorbent columns and preparative SDS-PAGE; the purified protein was then radiolabelled with Na^{125}I and treated with Endo-F. Separation of the Endo-F digestion products of the 20K T3 material by SDS-PAGE revealed that they contained only the 14K form and undigested 20K protein (Fig. 1a, lane 3). This supports the idea that the 23K/16K T3 species can be found only as an intermediate in the pulse-chase metabolic labelling experiments. The purified 14K and 20K proteins (Fig. 1b) were used for amino acid sequencing in the gas-phase sequencer. An unambiguous sequence was obtained only from the 14K material and was identical to the first 15 residues found by radiolabelled sequencing (Fig. 2). This is an important confirmation of the 14K T3 sequence determined with radiolabelled amino acids because it gives a unique contiguous sequence. Inspection of the limited N-terminal sequences of the 14K T3 polypeptide chain (δ -chain) did not reveal any homology with immunoglobulins¹⁵, T3/T-cell receptor α - or β -chains¹⁶⁻¹⁹, the T-cell differentiation antigen T8 (ref. 20) or rodent Thy-1 (ref. 21).

Structural differences between the T3 δ - and ϵ -chain were investigated further by HPLC of tryptic peptides. Radiolabelled 14K and 20K T3 polypeptide chains were prepared from HPB-ALL cells that had been cultured in the presence of ^3H -lysine or ^3H -arginine. For easy detection of the T3 proteins during the purification, ^{35}S -methionine was always added to the cultures. The tryptic peptide maps obtained from the T3 δ - and ϵ -chains were clearly different (Fig. 3). This finding, together with the N-terminal sequence data, confirms the existence of two distinct 20K T3 proteins.

The T3 ϵ -chain had previously been identified by its capacity to be labelled by the hydrophobic reagents ^{125}I -5-iodonaphthyl-1-azide (^{125}I -INA) (ref. 12) and ^{125}I -3-(trifluoromethyl)-3-(m-iodophenyl) deazirine (^{125}I -TID) (data not shown) and had been isolated based on its resistance to Endo-H and Endo-F. To obtain a means of positive identification of the T3 ϵ -chain, monoclonal antibodies were prepared by immunizing mice with

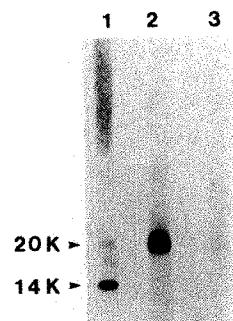


Fig. 4 Immunoblot analysis of T3 δ - and T3 ϵ -chains. Lane 1, rabbit anti-T3 δ -chain serum (6081); lane 2, monoclonal reagent SP6; lane 3, normal mouse serum.

Methods: Plasma membranes were isolated from HPB-ALL cells as described in Fig. 1 legend. Membrane proteins were treated with Endo-F and separated on a SDS-PAGE mini-gel²⁷. The proteins were transferred to nitrocellulose²⁹ and 4-mm wide strips were cut out and developed with antiserum 6081 or monoclonal antibody SP6 and ^{125}I -labelled protein A. Rabbit serum 6081 had been prepared with a synthetic peptide containing amino acids 1-14 of the T3 δ -chain (see Fig. 2 legend)³⁰. Monoclonal antibody SP6 was obtained by immunizing a BALB/c mouse with denatured 20K T3 material and selecting with purified δ - and ϵ -chains using a dot-blotting technique (S.P. *et al.*, manuscript in preparation).

purified denatured 20K T3 polypeptide chains. One of these antibodies (SP6) detected a 20K T3 polypeptide chains in an immunoblotting experiment after treatment with Endo-F (Fig. 4, lane 2). To establish the extent of hydrolysis with Endo-F, a control experiment was included using a rabbit antiserum (6081) raised against a synthetic peptide with the amino acid sequence 1-14 of the 14K T3 fragment. The material used in these immunoblotting experiments contained mainly the 14K T3 δ -chain and little glycosylated 20K T3 δ -chain (Fig. 4, lane 1). As no 14K T3 polypeptide chain was recognized by the mono-

clonal reagent SP6, we concluded that this reagent was specific for the T3 ϵ -chain. The experiment with the rabbit antipeptide antibody (6081) and SP6 also demonstrated that the N-terminal sequence of the 14K T3 (T3 δ -chain) cannot be found in the other 20K T3 structures (T3 ϵ -chain).

We conclude that the T3 ϵ -chain differs from the T3 δ -chain in several respects. First, peptide mapping, N-terminal sequences and the use of antibody reagents showed that their primary structures are unique. Second, labelling with ^{125}I NA¹² and ^{125}I -TID demonstrated that the T3 ϵ -chain binds these hydrophobic reagents much more strongly than the other T3 chains. In fact, the ϵ -chain contained at least 10 times more ^{125}I -TID than did the T3- δ -chain (data not shown). The observation that both hydrophobic nitrene- and carbene-containing reagents preferentially label T3 ϵ -chain supports the notion that this molecule has an extensive hydrophobic area. An alternative

possibility, that the T3 ϵ -chain contains amino acids that are highly reactive with both the nitrene and carbene reagent cannot be excluded. However, as anti-T3 and anti-T-cell receptor antibodies cause a rapid Ca^{2+} influx into the T cell, we have postulated previously that the T3 complex may contain a calcium channel²². The T3 ϵ -chain would be a good candidate for constituent of such an ion channel.

We thank Dr Robert Evans for a generous gift of the monoclonal reagent anti-Leu-4, Michael Raum, Robert Valas and Steve Meyers for technical assistance, Drs Peter Snow, Herge Spits and Jan de Vries for a critical review of the manuscript and Sharon McGrath for secretarial assistance. This work was supported by NIH grants AI-15066 and AI-17561. C.J. is a scholar of the Leukemia Society of America. J.B. is presently at the Netherlands Cancer Institute, Amsterdam, The Netherlands.

Received 12 August; accepted 9 October 1984.

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Cloning and expression of murine interleukin-1 cDNA in *Escherichia coli*

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Interleukin-1 (IL-1), a peptide hormone produced by activated macrophages, possesses the ability to modulate the proliferation, maturation and functional activation of a broad spectrum of cell types¹⁻⁷ and may play a major role in the initiation and amplification of immune and inflammatory responses through its action on these diverse cell populations⁸. IL-1 exhibits microheterogeneity in terms of its relative molecular mass (M_r , 13,000-19,000) and charge properties⁹, and although murine IL-1 has been purified^{9,10} and some of its basic structure-function relationships have been elucidated⁸, it has proved difficult to prepare sufficient amounts of IL-1 for direct and detailed sequence and structural studies. Here we report the cloning, sequence analysis and expression of murine IL-1 cDNA in *Escherichia coli*. The IL-1 cDNA codes for a polypeptide precursor of 270 amino acids. Biologically active IL-1 was produced in *E. coli* by expressing the carboxy-terminal 156 amino acids of the IL-1 precursor.

Recently, we reported the preparation of goat antibodies directed against murine IL-1¹⁰. Because of the specificity of these antibodies, we were able to develop an assay for the detection of IL-1 mRNA and analyse the primary translation form of IL-1 as well as the kinetics of its synthesis in activated macrophages¹¹. Poly(A)⁺ RNA was prepared from lysates of superinduced^{9,10} and uninduced P388D₁ cell line macrophages and translated in rabbit reticulocyte lysates. The translation

Table 1 Biological activity of recombinant IL-1

Preparation	μg Protein	$\Delta\text{c.p.m. } ^3\text{H-TdR}$ incorporation
Crude <i>E. coli</i> extract	0.7	26,370 $\pm$ 4,229
	1.4	57,637 $\pm$ 6,099
	2.8	63,940 $\pm$ 2,434
	7.0	93,475 $\pm$ 10,062
Purified recombinant IL-1	0.00002	9,997 $\pm$ 4,175
	0.00007	23,952 $\pm$ 3,733
	0.00014	46,561 $\pm$ 11,088
	0.00034	81,966 $\pm$ 2,087

E. coli containing pIL1 (1-156) were shifted to 42 °C for 3 h and the extracted with 7 M guanidine hydrochloride. An aliquot of the crude extract was diluted in phosphate-buffered saline and tested in the thymocyte proliferation assay¹⁰. ^3H -thymidine (TdR) incorporation in the presence of extracts of *E. coli* containing the expression plasmid with an insert in the opposite orientation was equivalent to background level (2,100 c.p.m.). The crude extract was concentrated by ammonium sulphate precipitation and then chromatographed on a 2.5 $\times$ 85 cm Ultrog AcA54 column. Fractions containing IL-1 activity were pooled and applied to an anti-IL-1 immunoadsorbent column¹⁰. The IL-1 was eluted with 4M KSCN and an aliquot was assayed for biological activity. On 12.5% SDS gels, the IL-1 migrated as a single band with M_r of 17,400

products were immunoprecipitated with anti-IL-1 IgG and analysed on SDS gels (Fig. 1). The messenger RNA from superinduced cells programmed the synthesis of a 33,000 M_r polypeptide which was immunoprecipitated with the anti-IL-1 antibodies (Fig. 1, lane 1), but not by normal goat IgG (Fig. 1, lane 3). Although present in markedly lower levels, the 33,000 M_r product was also synthesized in reticulocyte lysates supplemented with mRNA from uninduced cells (Fig. 1, lane 2). Purified low- M_r IL-1 blocked the immunoprecipitation of the 33,000 M_r peptide (Fig. 1, compare lanes 5 and 6), a finding that demonstrates the strong antigenic relationship between the two polypeptides. In parallel studies on the kinetics of IL-1 synthesis and secretion¹¹, we have established that the 33,000 M_r polypeptide is indeed a precursor for the low- M_r form of murine

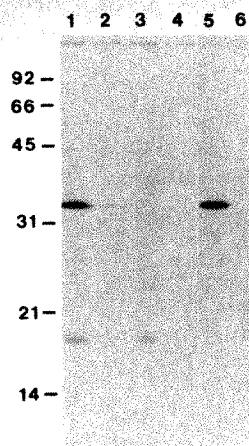


Fig. 1 Cell-free translation of P388D₁ IL-1 mRNA. Translation products were generated using rabbit reticulocyte lysates, immunoprecipitated with either normal goat IgG or anti-IL-1 IgG, and electrophoresed on SDS-polyacrylamide gels. Lanes 1 and 5, induced mRNA, translation products immunoprecipitated with anti-IL-1 IgG; lane 2, uninduced mRNA translation products immunoprecipitated with anti-IL-1 IgG; lane 3, induced mRNA, translation products immunoprecipitated with normal goat IgG; lane 4, uninduced mRNA, translation products immunoprecipitated with normal goat IgG; lane 6, induced mRNA, translation products immunoprecipitated with anti-IL-1 IgG in the presence of 2.5 µg of purified IL-1.

Methods: P388D₁ cells were incubated with 10^{-8} M mezerein and $10 \mu\text{g ml}^{-1}$ cycloheximide for 4 h^{9,10}. Poly(A)⁺ RNA was isolated from these superinduced cells as well as from uninduced P388D₁ cells using the guanidine thiocyanate-CsCl method²³ and oligo(dT)-cellulose chromatography²⁴. Each translation reaction contained 1 µg mRNA (1 µl), 20 µl rabbit reticulocyte lysate (Amersham, N.90), and 4 µl ³⁵S-methionine (Amersham SJ 204). The reaction mixtures were incubated for 1 h at 30 °C and then treated with 400 µg ml⁻¹ RNase and 20 mM EDTA for an additional 15 min. All subsequent manipulations were performed in the cold. Immunoprecipitation buffer (0.1 ml containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.35 M NaCl, 10 mM EDTA, and 25 mM Tris-HCl, pH 7.4) was added to each tube and the samples were 'precleared' with *Staphylococcus aureus*²⁵ (Pansorbin, Calbiochem) before overnight incubation with either normal goat IgG or anti-IL-1 IgG¹⁰. Immune complexes were precipitated using Pansorbin, extracted with 8 M urea, 1% SDS, 0.1 M dithiothreitol, 20 mM EDTA, and 50 mM Tris-HCl (pH 6.8) at 100 °C, and electrophoresed on 15% SDS-polyacrylamide gels. The gels were soaked in Enhance, dried and then exposed to X-ray film at -80 °C. *M_r* markers (shown $\times 10^{-3}$) were run in a parallel lane and visualized by Coomassie blue staining. In some experiments, a 18,000 *M_r* translation product was immunoprecipitated with normal and immune IgG. As purified IL-1 did not block the immunoprecipitation of this product, it represents nonspecific binding to goat IgG.

IL-1 which is normally isolated from the conditioned media of stimulated normal and cell line macrophages.

To enrich for the 33,000 *M_r* IL-1 mRNA, poly(A)⁺ RNA from induced P388D₁ cells was fractionated by centrifugation on a sucrose density gradient and analysed by cell-free translation (Fig. 2a). In this gradient, the mRNA coding for the 33,000 *M_r* IL-1 precursor exhibits a *M_r* slightly greater than *E. coli* 16S ribosomal RNA. Using this sucrose gradient enriched mRNA, a cDNA library of ~75,000 clones was constructed in pBR322 by established procedures¹². To identify an IL-1 cDNA clone, the library was randomly screened using the positive selection/translation analysis described by Parnes *et al.*¹³. After examining 150 pools (each containing 10 clones), a positive group was identified. One clone from this pool, termed pIL1 301, specifically hybridized to IL-1 mRNA (Fig. 2b).

Clone pIL1 301 contains a poly(A)⁺ tract and an insert of 701 base pairs (bp) (Fig. 3a). When used as a probe in a Northern blot against mRNA from induced P388D₁ cells, clone

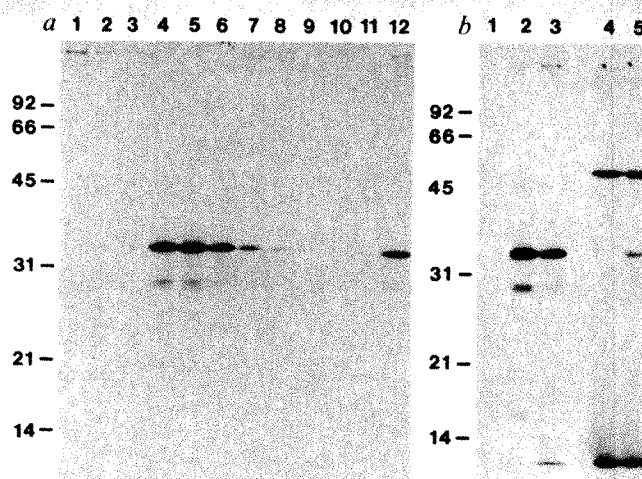


Fig. 2 Cloning of murine IL-1 cDNA. *a*, Translation and immunoprecipitation of polypeptides programmed by sucrose gradient fractionated P388D₁ mRNA. Lanes 1-11, 1 µg of each sucrose gradient fraction; lane 12, 1 µg of total P388D₁ poly(A)⁺ RNA. In a parallel gradient, *E. coli* 16S rRNA sedimented in the same fraction as the RNA sample used in lane 4, and 23S rRNA in the same fraction as the RNA sample used in lane 8. *b*, Translation and immunoprecipitation of polypeptides programmed by mRNA selected with clone pIL1 1301. Lanes 1 and 4, pBR322 selected mRNA; lanes 2 and 5, pIL1 1301 selected mRNA; lane 3, 1 µg of total P388D₁ poly(A)⁺ RNA. In lanes 4 and 5 a portion of the total translation products were run directly on the gel, whereas in lanes 1-3, the translation products were immunoprecipitated with anti-IL-1 IgG before electrophoresis.

Methods: *a*, Superinduced P388D₁ poly(A)⁺ RNA (0.7 mg) was fractionated on a 39-ml 5-25% sucrose gradient by centrifugation for 19 h at 28,000 r.p.m. in a SW28 rotor. Twenty gradient fractions were collected and the RNA was recovered by ethanol precipitation. The RNA from each of the 11 fractions comprising the middle portion of the gradient was examined for IL-1 programming activity in the *in vitro* translation assay (1 µg per reaction mixture). *b*, The fraction most highly enriched for the IL-1 mRNA (*a*, lane 5) was used to construct a cDNA library which was screened by hybrid-selection and cell-free translation as described by Parnes *et al.*¹³. After screening 1,500 clones (150 pools of 10), the candidate clone pIL1 1301 was compared with pBR322 in this assay. Plasmid DNA from 25 ml of bacterial cells was immobilized on nitrocellulose filters and hybridized to 20 µg of unfractionated induced P388D₁ mRNA. The specifically hybridized mRNA was eluted and translated as described in Fig. 1 legend. The 49,000 *M_r* product in lanes 4 and 5 is synthesized in the reticulocyte system in the absence of added RNA.

pIL1 1301 specifically hybridized to a single 2,000 nucleotide mRNA species (Fig. 4). A restriction fragment near the 5' end of the pIL1 1301 insert was used in a primer extension experiment to demonstrate that the majority of the IL-1 mRNA molecules possess a single 5' terminus, of which ~270 nucleotides are missing from clone 1301 (data not shown). To define the missing sequences, a specifically primed clone bank was screened to identify a clone, termed pIL1 31, which overlaps with clone 1301 (Fig. 3b) and might contain a complete copy of the 5' end of the mRNA. The nucleotide sequence (Fig. 3a) of clones 1301 and 31 predicts a single open reading frame, starting at the first AUG from the 5' end, coding for a protein of 270 amino acids. This is likely to be the complete IL-1 precursor, as there is an in-frame termination codon upstream from the putative initiation codon. Hence, murine IL-1 mRNA is ~1,974 nucleotides long (excluding the poly(A) tail), codes for a protein of 270 amino acids, and possesses a 5'-noncoding region of approximately 60 nucleotides and a 3'-noncoding region of 1,104 nucleotides (Fig. 3b). We have sequenced several additional IL-1 cDNA clones to verify the sequence presented in Fig. 3. Colony hybridizations against the oligo (dT)-primed library indicate that 1 in 2,000 clones contain IL-1 sequences. Assuming a 10-fold enrichment following sucrose density cen-

a

AAG TCT CCA GGG CAG AGA GGG AGT CAA CTC ATT GGC GCT TGA GTC GGC AAA GAA	54
ATC AAG ATG GGC AAA GTT CCT GAC TTG TTT GAA GAC CTA AAG AAC TGT TAC AGT	108
MET Ala Lys Val Pro Asp Leu Phe Glu Asp Leu Lys Asn Cys Tyr Ser	10
GAA AAC GAA GAC TAC AGT TCT GGC ATT GAC CAT CTC TCT CTG AAT CAG AAA TCC	162
Glu Asn Glu Asp Tyr Ser Ser Ala Ile Asp His Leu Ser Leu Asn Gln Lys Ser	20
TTC TAT GAT GCA AGC TAT GGC TCA CTT CAT GAG ACT TGC ACA GAT CAG TTT GTA	216
Phe Tyr Asp Ala Ser Tyr Gly Ser Leu His Glu Thr Cys Thr Asp Gln Phe Val	40
TCT CTG AGA ACC TCT GAA ACG TCA AAG ATG TCC AAC TTC ACC TTC AAG GAG AGC	270
Ser Leu Arg Thr Ser Glu Thr Ser Lys MET Ser Asn Phe Thr Phe Lys Glu Ser	60
CGG GTG ACA GTA TCA GCA ACG TCA AGC AAC GGG AAG ATT CTG AAG AAG AGA CGG	324
Arg Val Thr Val Ser Ala Thr Ser Ser Asn Gly Lys Ile Leu Lys Lys Arg Arg	80
CTG AGT TTC AGT GAG ACC TTC ACT GAA GAT GAC CTG CAG TCC ATA ACC CAT GAT	378
Leu Ser Phe Ser Glu Thr Phe Thr Glu Asp Asp Leu Gln Ser Ile Thr His Asp	90
CTG GAA GAG ACC ATC CAA CCC AGA TCA GCA CCT TAC ACC TAC CAG AGT GAT TTG	432
Leu Glu Glu Thr Ile Gln Pro Arg Ser Ala Pro Tyr Thr Tyr Gln Ser Asp Leu	110
AGA TAC AAA CTG ATG AAG CTC GTC AGG CAG AAG TTT GTC ATG AAT GAT TCC CTC	486
Arg Tyr Lys Leu MET Lys Leu Val Arg Gln Lys Phe Val MET Asn Asp Ser Leu	130
AAC CAA ACT ATA TAT CAG GAT GTG GAC AAA CAC TAT CTC AGC ACC ACT TGG TTA	540
Asn Gln Thr Ile Tyr Gln Asp Val Asp Lys His Tyr Leu Ser Thr Thr Trp Leu	150
AAT GAC CTG CAA CAG GAA GTA AAA TTT GAC ATG TAT GGC TAC TCG TCG GGA GGA	594
Asn Asp Leu Gln Gln Glu Val Lys Phe Asp MET Tyr Ala Tyr Ser Ser Gly Gly	170
GAC GAC TCT AAA TAT CCT GTT ACT CTA AAA ATC TCA GAT TCA CAA CTG TTC GTG	648
Asp Asp Ser Lys Tyr Pro Val Thr Leu Lys Ile Ser Asp Ser Gln Leu Phe Val	180
AGC GCT CAA GGA GAA GAC CAG CCC GTG TTG CTG AAG GAG TGG TCG GAA ACA CCA	702
Ser Ala Gln Gly Glu Val Leu Val Leu Lys Glu Leu Pro Glu Thr Pro	200
AAA CTC ATC ACA GGT AGT GAG ACC GAC CTC ATT TTC TTC TGG AAA AGT ATC AAC	756
Lys Leu Ile Thr Gly Ser Glu Thr Asp Leu Ile Phe Phe Trp Lys Ser Ile Asn	220
TCT AAG AAC TAC TTC ACA TCA GCT GCT TAT CCA GAG CTG TTT ATT GCC ACC AAA	810
Ser Lys Asn Tyr Phe Thr Ser Ala Ala Tyr Pro Glu Leu Phe Ile Ala Thr Lys	240
GAA CAA AGT CGG GTG CAC CTG GCA CGG GGA CTG CCC TCT ATG ACA GAC TTC CAG	864
Glu Gln Ser Arg Val His Leu Ala Arg Gly Leu Pro Ser MET Thr Asp Phe Gln	260
ATA TCA TAA AAG CAG CCT TAT TTC GGG AGT CTA TTC ACT TGG GAA GTG CTG ACA	918
Ile Ser	270
GTG TGT ATG TAC CAT GTA CAG GAA CCT TCC TCA CCC TGA GTC ACT TGC ACA GCA	972
TGT GCT GAG AGT CTG TAA TTC TAA ATG AGT GAT TAC ACT TGT TAT TTT AAA GAG	1026
GGA AAC CCT AGT GGA GCC ACC CCG ACA TAT GAT ACT ATC TGT TAT TTT AAA GAG	1080
TAC CCT ATA GTT TGC TCA GTA CTA ATC ATT TTA ATT ACT ATT CTG CAT GGC ATT	1134
CTT AGG AGG ATC AAA AAG ACT CTA CAC ATA TTA CAG ATG GGT TAA CAA AGG GAT	1188
AAA ACA ACT GAA AAG CAC ACT CAA TGC ATT TGG AAT ATA AAT TCA CAG ACC AAT	1242
CTC ACT GTG CAC CTT CGG CTT CAA AAT GCC AGT TGA GTA GGA TAA AGG TAT AAG	1296
AAC TTA ATG CTG TCA TTT TCA AAA GGA AGG GGA CAG TAG CTA CAT CTT TCC TAC	1350
CTC AGT GGG TTT TAC TCC AGT GAG ATC ATT TGG ATG AAA TCC TCC TGT AAC AGA	1404
CCT CAA GGA GGA GAC AGA CTG TTG AAT GTT ATT TTT AAG TTA TTT TAT ATA TGT	1458
ATT TAT AAA TAT ATT TAT GAT AAT TAT ATT TAT TAT GGA ACA TCC TTA AAT CCT	1512
CTG AGC TTS ACA GGC ATC CTC ACA GCA GGA TTT TCT AGG TGG TCA GTT AGA TAT	1566
AGT TTC CTC TAG AGC ACC ATG CTA CAG ACT TTA CAC TTT TTC CAC AGC CAC GAA	1620
GCT CTC TGT ACA TTC CTG TAC TTG GGA GCC CTT TCA TCA TGA TCT TAA TCT GTA	1674
CTG TTT ACT TTG TTC ATC TAA AAT GAT AAT GAT GTC AGT CTT TTT CCC TCC CAT	1728
CCT TAA AGC TGT CTG GGT ATT CTT ACA TCA TTT AGT CTC ACC TGT AAC TAA CAC	1782
CAA CCA TCT AAA GAT GGA AAG AGC TTA ACT GTG ACA ACC ACA TCA CTG TTA CCT	1836
GAA GTT TCT TTT CTA GAA TGT AAT CAG TGT TCT CCC TGG ATT CCA ATT TTT TTT	1890
TCA AAC CAC AGT ATC ATG TAA CTA TCA ACA ATA ACA ATC ACA TTA TTA TTA	1944
ATC ATA ATT AAA TAA AAC AAG TTT GAG CTG AAA AAA AAA AA	1974

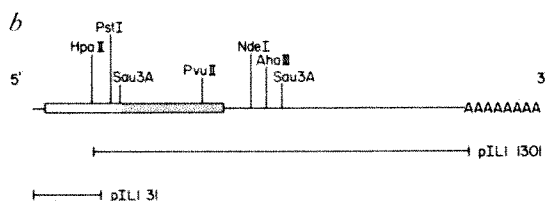


Fig. 3 Nucleotide sequence of IL-1 precursor cDNA and the predicted amino acid sequence of the IL-1 precursor. **a**, The nucleotide sequences of clones pIL1 1301 and pIL1 31 were determined by the chemical sequencing method²⁶. Nucleotide numbers are shown along the right-hand side of the sequence and the predicted amino acid sequence and corresponding position numbers are shown beneath. Clone pIL1 31 was isolated by using the oligonucleotide 5'-GTGAAGGTCTCACTGAAACTCAGCCG-3', the sequence of which is derived from clone 1301 and complementary to positions 322-347 of the IL-1 mRNA, to generate and screen a specifically-primed library from sucrose- gradient enriched P388D₁ mRNA. **b**, Diagram of mouse IL-1 mRNA shows that clone 31 extends from nucleotide 1-302 and that 1301 extends from nucleotides 269-1,974 plus poly(A) tract. The boxed area indicates the position of the coding region for the 270-amino acid precursor and the stippled region indicates that portion containing IL-1 activity. For orientation purposes, certain unique restriction enzyme cleavage sites are indicated. The *Sau3A* sites are not unique but are included to indicate the position of the 740-bp fragment that was used for *E. coli* expression.

Fig. 4 Northern blot analysis of IL-1 mRNA. Five μ g of induced poly(A)⁺ RNA was electrophoresed on a 1.4% glyoxal-agarose gel, transferred to nitrocellulose²⁷ and probed with nick-translated clone pIL1 1301. *M_r* markers (shown in kbp) were run in a parallel lane and visualized by ethidium bromide staining.

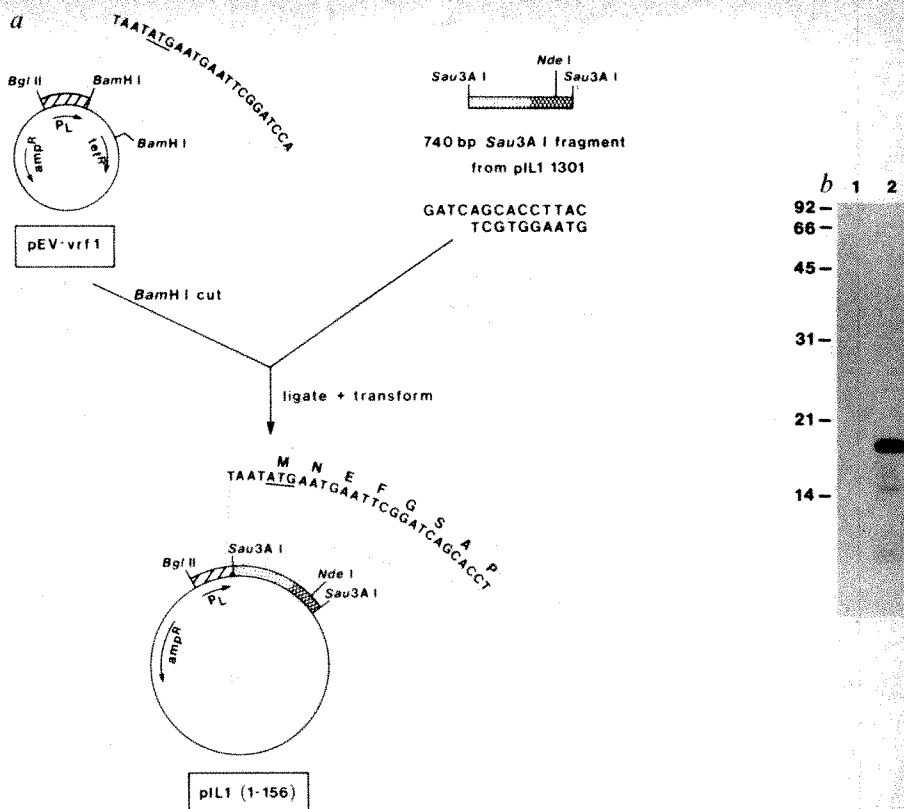
2.9 —
1.5 —
1.3 —
1.1 —
0.9 —
0.6 —

trifugation and equal cDNA cloning efficiencies, IL-1 mRNA accounts for 0.005% of the total poly(A)⁺ RNA from superinduced P388D₁ cells.

We have used two approaches to the mapping of the sequence responsible for IL-1 activity within the 270-amino acid precursor. Purified low-*M_r* P388D₁-derived IL-1 exhibits microheterogeneity on both Tris-glycinate and SDS-polyacrylamide gels^{10,14}. Attempts to obtain amino-terminal sequence data on this mixture were unsuccessful. However, when such IL-1 preparations were treated with cyanogen bromide, a number of peptides were released, of which two were isolated by means of HPLC in sufficient quantities for sequencing: peptide 1 $\rightarrow$ Lys-Leu-Val-Arg-Gln-Lys and peptide 2 $\rightarrow$ Ser-?-?-Tyr-Thr-Gln-?-Asp-Leu. Both of these sequences are found in the 170-amino acid precursor as deduced from the cDNA sequences (Fig. 3a). The sequence of peptide 1 is located at positions 130-135 and is preceded by a Met at position 129. The sequence of peptide 2 is located at positions 115-124, but is not preceded by a Met residue. We infer that peptide 2 is released by cyanogen bromide cleavage from the amino-terminal end of a subset of the IL-1 molecules that begin at the Ser residue at position 115.

We have also used expression in *E. coli* to prove that IL-1 is contained within the carboxy-terminal region of the 270-amino acid precursor. A 740-bp *Sau3A* fragment, beginning with the amino acid sequence of peptide 2 and containing the sequence coding for the last 156 amino acids of the 270-amino acid precursor, was isolated from pIL1 1301 and inserted into the *Bam*HI site downstream from an initiation codon in an *E. coli* expression plasmid (Fig. 5a). Expression in this plasmid (R. Crowl, manuscript in preparation) is controlled by a promoter regulated by a temperature-sensitive repressor. Guanidine hydrochloride (7 M) extracts of cells containing the recombinant plasmid were assayed for IL-1 activity in the murine thymocyte proliferation assay and for IL-1 immunoreactivity using goat anti-IL-1 IgG. Several clones containing the insert in the incorrect orientation made no detectable IL-1 activity or protein (Fig. 5b) at either 30 °C or 42 °C. Clones containing the insert in the correct orientation, pIL1 (1-156), made no IL-1 at 30 °C, but at 42 °C they synthesized a 17,400 *M_r* polypeptide that reacted with anti-IL-1 IgG (Fig. 5b) and was active in the thymocyte proliferation assay (Table 1). Crude extracts of pIL1 (1-156) contained 10⁷ units of IL-1 activity per gram of the recombinant clone. Using gel filtration chromatography and an anti-IL-1 immunoadsorbent, we have purified the recombinant IL-1 and have found that it possesses the same specific activity as the IL-1 released by P388D₁ cells ($\sim 6 \times 10^6$ units per mg, Table 1). The results of preliminary experiments indicate that the recombinant IL-1 possesses the same range of biological activities as the natural IL-1 produced by murine macrophages.

Fig. 5 Expression of IL-1 in *E. coli*. *a*, Expression clone pIL1 (1-156) was constructed by inserting a 740-bp *Sau*3A fragment from plasmid pIL1 1301 into the *Bam*HI site of pEV-vr1 (ref. 15) and transforming *E. coli* strain RR1 containing the compatible plasmid pRK248 cIts (ref. 28). Orientation of the insert in each clone was determined by the location of the *Nde*I site in the 3'-noncoding region of the IL-1 fragment. As indicated by the sequence around the initiation codon of the expression plasmid, this construction adds five additional amino acids to the amino-terminus of the expected 156 amino acid IL-1 polypeptide. *b*, *E. coli* containing the expression plasmid with an insert in the opposite orientation (lane 1) and in the correct orientation (lane 2) were grown in M9 media lacking methionine at 30 °C. When A_{550} reached 0.3, the cultures were shifted to 42 °C for 75 min. Forty μ Ci of ^{35} S-methionine were added to 0.5 ml of each culture and incubated for an additional 5 min at 42 °C. The bacteria were pelleted, solubilized in 25 μ l of 7 M guanidine hydrochloride and resuspended in 0.5 ml of immunoprecipitation buffer. Immunoprecipitation and gel analysis were performed as described in Fig. 1 legend.



The sequence of the cloned cDNA (Fig. 3a) and its expression in *E. coli* offers, for the first time, an appreciation of the primary structure of IL-1 and its precursor. The 270-amino acid precursor has a calculated M_r of 31,026, which is reasonably close to the estimated size (33,000) of the cell-free translation product (as assessed by SDS-gel electrophoresis). The precursor contains two cysteine residues (positions 14 and 47), three potential *N*-glycosylation sites (positions 66, 141 and 145) and an unusual tetrabasic region, Lys-Lys-Arg-Arg (positions 85-88). Homology searches of the GenBank Nucleic Acid Database and the NBRF Protein Sequence Database demonstrate that the sequence of the mouse IL-1 gene and protein have not previously been described. The sequence of IL-1 was compared with that of several other lymphokines, interleukin-2¹⁵, interleukin-3¹⁶, γ -interferon¹⁷, and granulocyte-macrophage colony-stimulating factor¹⁸, and no sequence homologies were evident. There is some weak homology ($\sim 32\%$) between the first 100 amino acids at the amino-terminal end of the mouse IL-1 precursor and the carboxy-terminal 100 residues of human and baboon α_1 -antitrypsin¹⁹. However, there is no homology with antithrombin III and ovalbumin, the other two members of this gene family^{19,20}.

The 270-amino acid precursor does not seem to contain a classic signal peptide. Hydropathicity plots²¹ indicate that the precursor is mostly hydrophilic—there is no sizeable hydrophobic region, especially at the amino-terminus. In addition, *in vitro* translation experiments using rabbit reticulocyte lysates and dog pancreas microsomes have not revealed any processing of the 33,000 M_r IL-1 precursor (data not shown). Furthermore, we have detected¹¹ the 33,000 M_r form of IL-1 in the culture fluid of stimulated macrophages—a finding that raises the interesting possibility that the 33,000 M_r peptide may be the species of IL-1 secreted by activated cells.

The carboxy-terminal 156-amino acid polypeptide that is active in the thymocyte proliferation assay has a calculated M_r of 17,992. The absence of cysteine residues in the low- M_r IL-1 provides an explanation for the insensitivity of IL-1 to reduction and alkylation⁸. Chou-Fasman calculations²² on this polypeptide predict a structure with extensive β -sheet character and little α -helicity.

In conjunction with other recent studies on IL-1 synthesis¹¹, the cloning, sequence analysis and expression of IL-1 cDNA clearly demonstrates that IL-1 from normal as well as cell-line macrophages is initially synthesized as a 270-amino acid precursor. Increased IL-1 mRNA levels (Fig. 1) probably indicate that this precursor is expressed in higher levels when the macrophage is stimulated. In some as yet unknown fashion, the IL-1 precursor is converted to the low- M_r form normally detected in the culture fluid of activated macrophages. Although additional studies remain to be done, we favour the hypothesis that the 33,000 M_r precursor is secreted and then enzymatically converted to a lower- M_r form. We suggest that one or more proteases released by the macrophage may be responsible for cleaving at a primary processing site (perhaps the tetrabasic Lys-Lys-Arg-Arg at position 85-89). Subsequent proteolytic activity would generate 'ragged' amino-termini, thus providing an explanation for the M_r and charge microheterogeneity that is characteristic of IL-1.

We thank Jean Severin, Malcolm Newton, Stephan McAndrew, Alvin Stern, Philip Familletti, James Jenson, Mike Vitek, Russell Blacher, May Chang, Janusz Wideman, Patrick Trown, Ann Skalka and L. Patrick Gage for advice and support. This research was supported in part by NSF grant PCM-8110370 (to S.B.M.).

Received 30 July; accepted 9 October 1984.

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Mitogens increase phosphorylation of phosphoinositides in thymocytes

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In many cell systems the interaction of ligands with their receptors causes rapid breakdown and resynthesis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂)¹⁻⁵. Recent work has focused on the role of the degradation products of PtdIns(4,5)P₂ as intermediates in the activation of cell function and growth⁶: inositol trisphosphate (InsP₃) can release Ca²⁺ from intracellular stores⁷⁻⁹ and diacylglycerol is thought to activate protein kinase C^{6,10}. This enzyme is also activated by phorbol esters (for example, 12-O-tetradecanoyl phorbol 13-acetate, TPA)¹¹ and this is assumed to account for the pleiotropic effects of TPA on cell function and growth. Mouse thymocytes are not mitogenically stimulated by TPA alone, but it is a potent co-mitogen in combination with either concanavalin A (Con A) or A23187 (A. N. Corps and J.C.M., unpublished observations). Here we show that mitogenic concentrations of TPA, A23187 and Con A¹² each cause an increase in the net phosphorylation of phosphatidylinositol (PtdIns) to PtdIns(4,5)P₂ in mouse thymocytes. This is consistent with stimulation by the mitogens of the same phosphoinositide phosphorylations in intact cells as recently demonstrated for the isolated products of the *src* and *ros* viral oncogenes in a cell-free system^{13,14}.

When mouse thymocytes were labelled with myo-[2-³H]inositol¹⁵, the amounts of ³H-label in each of the phosphoinositides increased at an approximately constant rate for 12-14 h, after which there was no further increase in incorporation. We assume, therefore, that the phosphoinositides are at isotopic equilibrium with ³H-inositol after 14 h and that subsequent changes in the amounts of ³H-PtdIns, ³H-PtdIns(4)P (phosphatidylinositol 4-phosphate) and ³H-PtdIns(4,5)P₂ reflect changes in the concentrations of these lipids.

TPA (10 nM) and A23187 (100 nM) stimulated increases in PtdIns(4)P and PtdIns(4,5)P₂ within 5 min and the amounts of ³H-label in both polyphosphoinositides continued to rise for ~15 min (Fig. 1a, b). Con A (1 µg ml⁻¹) caused a small, but variable, decrease in PtdIns(4,5)P₂ which amounted to typically 10% after 1 min, followed by a variable increase of up to 25% above the initial level after 10 min. In most experiments there was no initial decrease in PtdIns(4)P after Con A addition, and the amount of PtdIns(4)P increased by 30-60% after 10 min (Fig. 1a, b). The increased steady-state levels of the polyphosphoinositides stimulated by each of the mitogens were similar; these levels may be regulated in part by feedback inhibition of the phosphoinositide kinases by the polyphosphoinositides^{13,14,16}. There was no change in the amount of PtdIns over the time course of these responses (see Fig. 1 legend), thus it is clear that either the phosphoinositide kinases that synthesize PtdIns(4,5)P₂ from PtdIns via PtdIns(4)P (refs 1, 17) were

activated or that breakdown of PtdIns(4,5)P₂ was inhibited. This phospholipid is catabolized by a phosphodiesterase to release InsP₃ and diacylglycerol, and by a phosphomonoesterase to release PtdIns(4)P and phosphate¹⁸⁻²⁰.

Figure 1c, d shows the effects of the mitogens on the breakdown of PtdIns(4,5)P₂ by phosphodiesterase: neither TPA nor A23187 had any effect (±10%) on the amounts of either InsP₃ or the other inositol phosphates within 15 min. However, there was an increase in the level of InsP₃ within 30 s of the addition of Con A (Fig. 1c) and later increases in inositol bisphosphate (InsP₂) and inositol phosphate (InsP)¹⁵. The levels of InsP₃ were increased approximately sixfold after 5-8 min, then declined slowly. The stimulated rates of InsP₃ release and breakdown to InsP₂ and InsP must therefore be similar, as the total amount of inositol phosphates continued to increase for at least 15 min (Fig. 1d). Furthermore, the total amount of ³H-labelled inositol phosphates at 15 min was approximately twice that of ³H-labelled PtdIns(4,5)P₂ before the addition of Con A. The comparison is only approximate because the inositol phosphates recovered underestimate the extent of PtdIns(4,5)P₂ breakdown (some InsP is degraded to inositol even in the presence of Li⁺), and the percentage recovery of the polyphosphoinositides¹⁸ may be lower than that of the inositol phosphates (>85%; ref 15). Nevertheless, it is clear that Con A must cause a large compensating stimulation of polyphosphoinositide synthesis to account for the amounts broken down to inositol phosphates at 20 min. Con A, TPA and A23187 all stimulate net polyphosphoinositide synthesis at a similar rate (Fig. 1a, b). However, the actual rate of PtdIns(4,5)P₂ synthesis stimulated by Con A is at least 10-fold greater when the coincident stimulation of breakdown to inositol phosphates is taken into account. The data clearly indicate that Con A, but neither TPA nor A23187, activates polyphosphoinositide phosphodiesterase. The increase in PtdIns(4)P and PtdIns(4,5)P₂ stimulated by each of the three mitogens must, therefore, be due to either activation of the phosphoinositide kinases or inhibition of the phosphomonoesterases.

Although neither A23187 nor TPA had any significant effect on the amount of InsP₃ in the cells, they had opposing effects on the steady-state increase in InsP₃ stimulated by Con A (Fig. 1c), which can be analysed into effects on polyphosphoinositide synthesis and breakdown. TPA potentiated the steady-state increase in InsP₃ stimulated by Con A, although the initial rates of InsP₃ production were similar with and without TPA (Fig. 1c). This effect on InsP₃ production is most simply explained by the increase in the levels of PtdIns(4)P and PtdIns(4,5)P₂ in response to TPA plus Con A (not shown) compared with Con A alone, and any effect of TPA on the activated polyphosphoinositide phosphodiesterase or the inositol phosphate phosphatases appears to be small. In contrast to TPA, A23187 inhibited InsP₃ production by Con A (Fig. 1c) and as net polyphosphoinositide synthesis is stimulated by A23187 (Fig. 1a, b), the reduction in inositol phosphate levels may be caused either by a Ca²⁺-mediated inhibition of the activated polyphosphoinositide phosphodiesterase, or by stimulation of the inositol phosphate phosphatases.

Further indication of the mechanisms by which the synthesis and breakdown of PtdIns(4,5)P₂ can be altered was obtained by examining the effects of sodium azide and cyclic nucleotide analogues. When thymocytes were incubated for 10 min with sodium azide (10 mM), their ATP content was reduced from 3 mM to 0.75 mM; this reduced the amounts of PtdIns(4)P and PtdIns(4,5)P₂ by 15-20%, but had a negligible effect on the inositol phosphates in unstimulated cells (Fig. 2). However, azide reduced the stimulation of net polyphosphoinositide synthesis by the mitogens (shown for Con A in Fig. 2a, b). Azide also reduced the steady-state level of InsP₃ in Con A-stimulated cells, but not the initial rate of InsP₃ release (Fig. 2c). These effects can be attributed to a reduction in the phosphorylation of the polyphosphoinositides and are qualitatively similar to those reported in slices of parotid gland treated with 2,4-dinitrophenol³.

Fig. 1 Stimulation of polyphosphoinositide synthesis (*a, b*) and breakdown (*c, d*) by mitogens. Thymocytes from 4–6-week-old BALB/c mice were prepared in an inositol-free culture medium and labelled for 18 h with *myo*-[2-³H]inositol as described previously¹⁵. The cells were washed twice by centrifugation in Earle's balanced salts solution buffered with 10 mM HEPES (supplemented with 5 mM LiCl^{30,31} in experiments where inositol phosphates were assayed) and incubated (2.5×10^6 cells in 0.5 ml per sample) for 15 min at 37 °C. In each experiment the data for the phosphoinositides were obtained from triplicate determinations with a standard deviation $\leq 10\%$ of the mean. The data points for the inositol phosphates are single determinations, but are typical of data obtained in at least three experiments. *a, b*, At zero time the following additions were made: 10 nM TPA ($\square$), 100 nM A23187 (Δ), 1 $\mu\text{g ml}^{-1}$ Con A ($\bullet$), or salts solution ($\circ$). The reactions were stopped by adding 1.88 ml chloroform/methanol (1:2), the phosphoinositides were extracted in acidic conditions, deacylated with a methylamine reagent and the glycerophosphorylinositides separated by anion-exchange chromatography¹. The amounts of PtdIns(4,5)P₂ (*a*) and PtdIns(4)P (*b*) are expressed as percentages of the amounts in untreated cells (3,280 and 1,080 d.p.m. per 10^7 cells, respectively), which were constant during the experiment. There was no change in the amount of PtdIns (190,000 d.p.m. per 10^7 cells) in unstimulated or mitogen-treated cells (TPA, A23187, Con A: $100.6 \pm 4.4\%$ of control ($n=6$), 98.1% ($n=2$) and $100.9 \pm 1.4\%$ ($n=6$) respectively). The data are from typical experiments: the mean values ($\pm$ s.d.) for PtdIns(4,5)P₂ 10 min after treatment with TPA, A23187 and Con A were $135.6 \pm 22.5\%$ ($n=6$), 122.4% ($n=2$) and $105.7 \pm 18.2\%$ ($n=6$) respectively; those for PtdIns(4)P were $192.1 \pm 36.8\%$ ($n=6$), 204.4% ($n=2$) and $139.3 \pm 16.1\%$ ($n=6$) respectively. *c, d*, Polyphosphoinositide breakdown to inositol phosphates. 10 nM TPA ($\square$, $\blacksquare$), 100 nM A23187 (Δ , $\blacktriangle$) or salts solution ($\circ$, $\bullet$) were added (open arrow) and the cells incubated for 10 min before addition of 1 $\mu\text{g ml}^{-1}$ Con A to some aliquots of the cells as indicated by the closed symbols. The inositol phosphates were assayed as described elsewhere^{15,32,33} and the amounts of InsP₃ (*a*) and the sum of InsP, InsP₂ and InsP₃ (*b*) are expressed as percentages of the amounts in control cells (1,170, 180 and 360 d.p.m. per 10^7 cells, respectively). Note that A23187 (100 nM) had no effect on the ATP content of thymocytes (measured as in ref. 34) during the experiments shown.

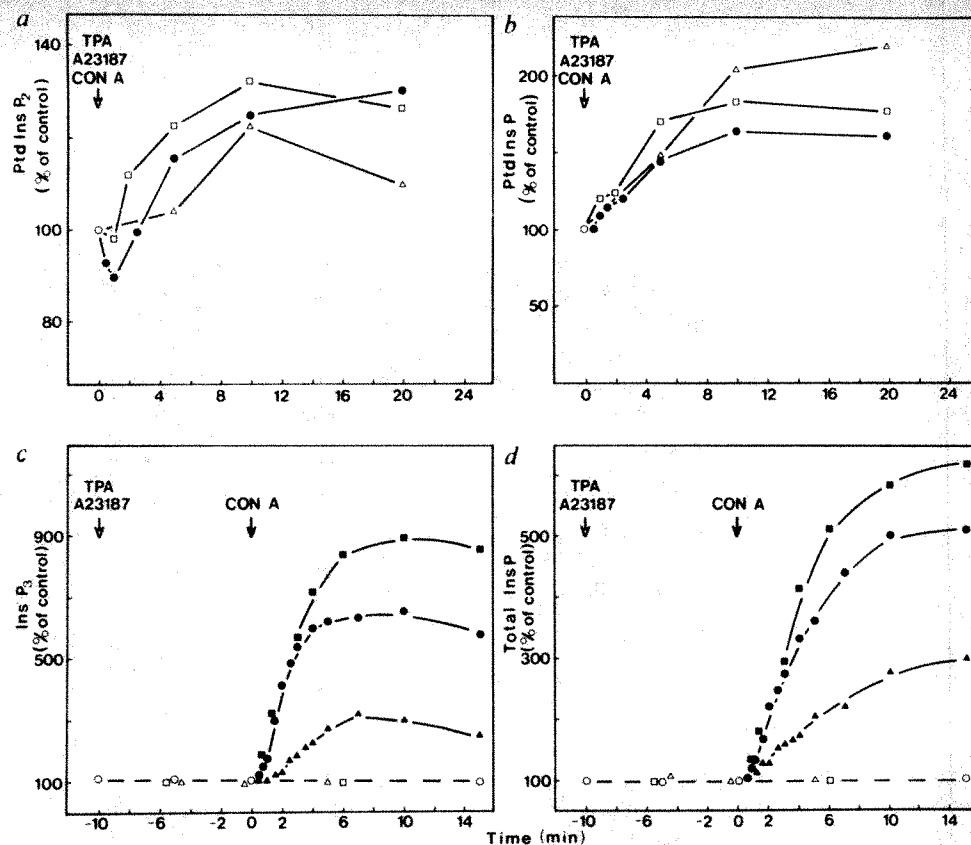
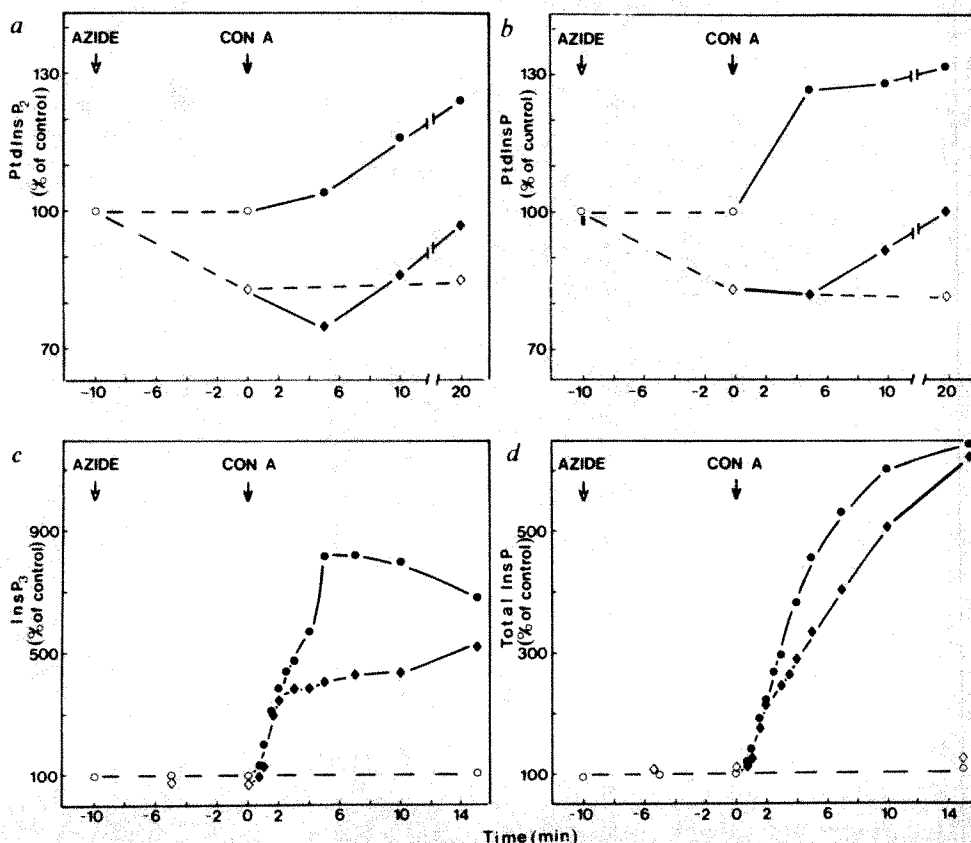


Fig. 2 The effect of azide on polyphosphoinositide synthesis (*a, b*) and breakdown (*c, d*). Experimental conditions were as described in Fig. 1 legend. Sodium azide (10 mM; $\diamond$, $\blacklozenge$) or salts solution ($\circ$, $\bullet$) was added at the times indicated by the open arrow and after 10 min Con A (1 $\mu\text{g ml}^{-1}$) was added to some aliquots of cells (closed symbols). *a, b*, Polyphosphoinositide data, average of two experiments. *c, d*, Production of InsP₃ and total inositol phosphates.



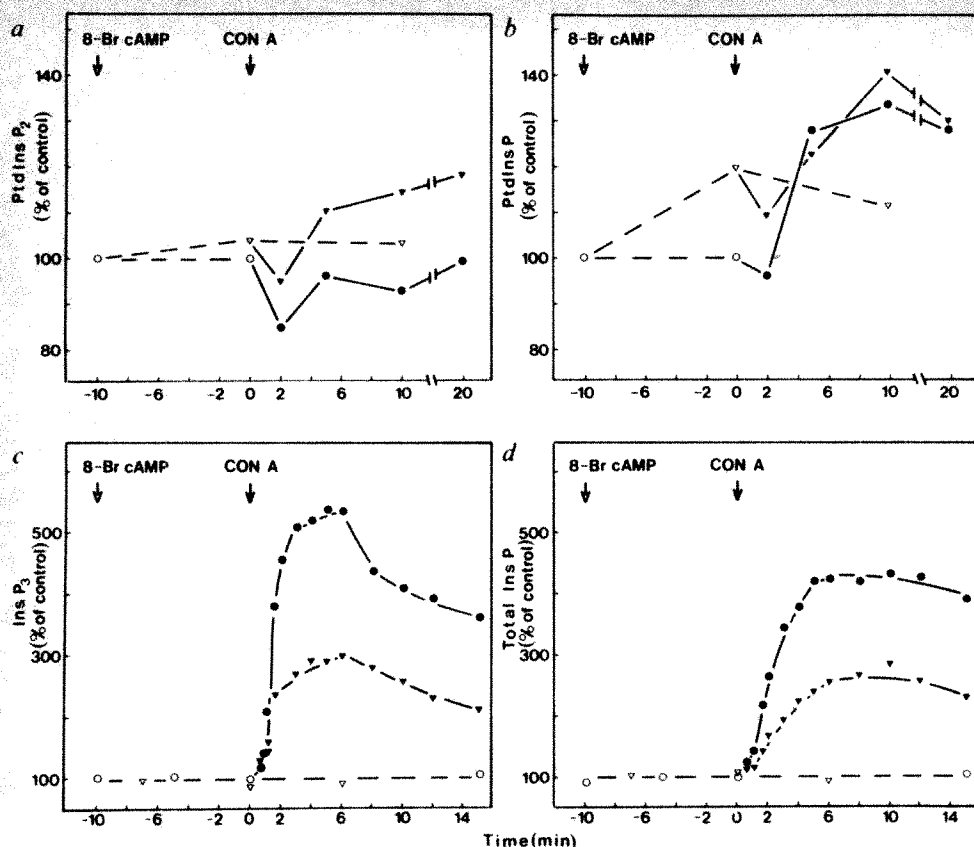


Fig. 3 The effect of 8-Br-cAMP on polyphosphoinositide synthesis (a, b) and breakdown (c, d). The experimental conditions were as described in Fig. 1 legend. 8-Br-cAMP (10 mM; ∇ , $\blacktriangledown$) or salts solution ($\circ$, $\bullet$) was added at the time indicated by the open arrow and after 10 min Con A ($1 \mu\text{g ml}^{-1}$) was added to some aliquots of cells (closed symbols). a, b, Polyphosphoinositide data, average of three experiments. 10 min after 8-Br-cAMP addition (no Con A), PtdIns(4,5)P₂ = $103.8 \pm 5.5\%$ of control, PtdIns(4)P = $119.7 \pm 6.2\%$ of control; in the same experiments, PtdIns = $105.0 \pm 7.2\%$. c, d, Production of InsP₃ and total inositol phosphates.

There have been several reports that cyclic nucleotides inhibit the phosphoinositide responses to ligands in a variety of cells²¹⁻²³. We have used 8-bromocyclic AMP (8-Br-cAMP), a membrane-permeant analogue of cyclic AMP, as thymocytes become desensitized to hormones which elevate cyclic AMP after prolonged incubation at 37 °C as required in these experiments (ref. 24 and J.P.M., unpublished observations). 8-Br-cAMP (10 mM for 10 min) increased the amount of PtdIns(4)P by ~20%, but had no effect on PtdIns(4,5)P₂ (Fig. 3a, b). However, in cells stimulated with Con A, the amount of PtdIns(4,5)P₂ was increased in the presence of 8-Br-cAMP, although there was little effect on PtdIns(4)P. 8-Br-cAMP (but not 8-bromocyclic GMP; see ref. 23) inhibited production of inositol phosphates in response to Con A, although there was no effect ($\pm 10\%$) on inositol phosphate levels in the absence of Con A (Fig. 3c, d). The initial rate of InsP₃ production in response to Con A was not affected greatly by 8-Br-cAMP, but the steady-state level was reduced. These results suggest that 8-Br-cAMP has a small stimulatory effect on net polyphosphoinositide synthesis (as reported previously^{25,26}) and are also consistent with inhibition by 8-Br-cAMP of polyphosphoinositide phosphodiesterase after it has been activated by Con A. An alternative possibility is that 8-Br-cAMP activates the inositol phosphate phosphatases. The effects of 8-Br-cAMP on Con A-stimulated InsP₃ production are therefore similar to those of A23187, but their mechanisms of inhibition may differ because there are kinetic differences in their effects (see Figs 1c, d, 3c, d).

It is apparent from the experiments described here that each mitogen tested increases net polyphosphoinositide synthesis; whether this is mediated by stimulation of the phosphoinositide kinases or by inhibition of the phosphomonoesterases is unresolved. However, regulation of these enzymes clearly can occur via Ca²⁺-dependent (A23187) or Ca²⁺-independent (TPA) mechanisms, as TPA does not raise the intracellular free Ca²⁺ concentration in thymocytes²⁷. Con A stimulates the production of InsP₃, which may be involved in generating the rise in Ca²⁺ in thymocytes in response to Con A^{27,28}, and the Ca signal may in turn stimulate polyphosphoinositide synthesis. Con A maintains prolonged production of inositol phosphates by an

approximate balance in the stimulation of both the synthesis and breakdown of PtdIns(4,5)P₂. Con A also causes the production of diacylglycerol²⁹, which may activate protein kinase C and hence stimulate net polyphosphoinositide synthesis by analogy with the effect of TPA. It is possible, therefore, that both products of PtdIns(4,5)P₂ breakdown act as feedback activators of its synthesis. It should, however, be emphasized that there is at present no direct proof that diacylglycerol produced by PtdIns(4,5)P₂ breakdown activates protein kinase C in intact cells, or that the small (less than threefold) rise in the Con A-induced intracellular Ca²⁺ concentration is sufficient to stimulate polyphosphoinositide synthesis. It is important to establish whether either of these mechanisms is operating, as they may occur in a wide range of cells. What is of interest, irrespective of mechanism, is that each of the mitogens stimulates the net phosphorylation of the phosphoinositides in lymphocytes. This finding may be compared with the recent observation that viruses carrying the *src* (ref. 13) and *ros* (ref. 14) oncogenes stimulate phosphoinositide phosphorylation in fibroblasts, which can be attributed to the phosphoinositide kinase activity of the isolated gene products.

M.V.T. holds a MRC Studentship. This work was funded by a grant from the SERC to J.C.M. Technical assistance was provided by Mr R. W. Plumb and some of the preliminary experiments were performed by Miss S. Crank.

Received 18 July; accepted 27 September 1984.

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Two steroid 21-hydroxylase genes are located in the murine *S* region

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A common inherited disorder of steroidogenesis in man, 21-hydroxylase (21-OH) deficiency, is linked to the *HLA* major histocompatibility complex (MHC)¹, and is associated in particular with certain allotypes of the *HLA*-linked complement proteins^{2,3}. Recently, this disorder was demonstrated to result from a defective structural gene for the 21-OH enzyme, also termed cytochrome P-450_{C21} (ref. 4). The human (*HLA*) and murine (*H-2*) MHCs are homologous in overall organization and in the structures of their component genes⁵. To determine whether 21-OH genes are located in the *H-2* complex, we have now used a bovine adrenal complementary DNA clone encoding part of 21-OH⁶ to examine a cluster of overlapping cosmid clones derived from the *S* region of the BALB/c mouse⁷. We found that there are two 21-OH genes in this region, located immediately 3' to the *C4* and *Slp* genes.

The cDNA clone used in this study, pC21a, contains a 520-base pair (bp) insert in the *Pst*I site of pBR322, encoding approximately the middle third of the bovine 21-OH peptide⁶. To ascertain whether mouse genomic DNA contained a homologue of bovine 21-OH, the 430-bp *Pst*I fragment of pC21a was hybridized to Southern blots⁸ of BALB/c DNA (Fig. 1a). An *Eco*RI fragment of 3.0 kilobases (kb) and a 3.7-3.8-kb *Bam*HI fragment hybridized with the probe. In some experiments, the *Bam*HI band was resolved as a doublet.

Mapping of the chromosomal location of these genes was guided by linkage data obtained in man. Patients carrying 21-OH deficiency on the haplotype *HLA-Bw47; DR7* also carry a null allele at one of the two *C4* loci encoding the fourth component of complement in man^{2,3}. This haplotype invariably carries a deletion of a 21-OH structural gene, suggesting that the 21-OH and *C4* loci are adjacent and have both been deleted in this haplotype⁴. In man, both the *C4A* and *C4B* loci encode

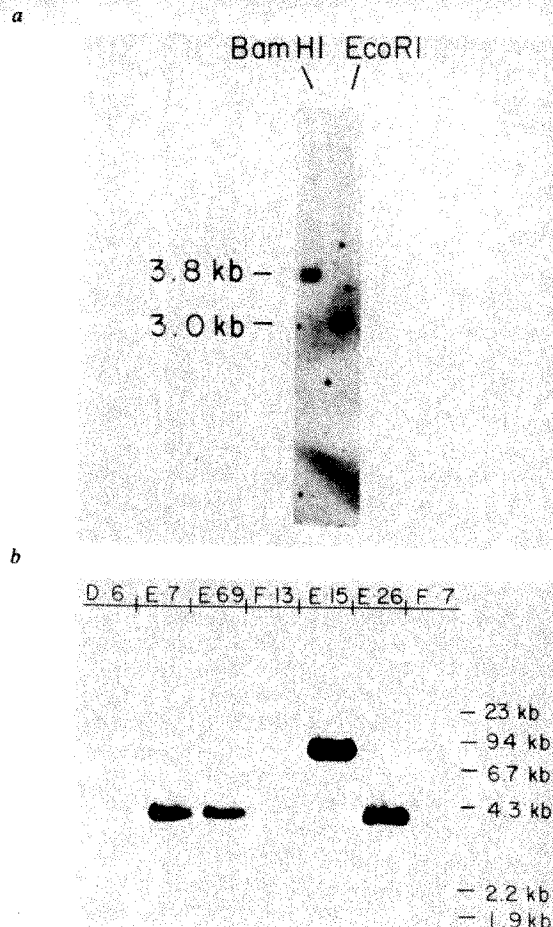


Fig. 1 Hybridization of pC21a to BALB/c mouse genomic DNA (a) and cosmid clones (b). **Methods:** a, 10 µg of DNA prepared from mouse liver were digested overnight with 4 U µg⁻¹ of the indicated restriction enzyme. The digests were subjected to electrophoresis in 1.0% agarose and blotted to nitrocellulose⁸. The 430-bp *Pst*I fragment of pC21a⁶ was radioactively labelled with ³²P-dCTP to a specific activity of 10⁹ d.p.m. µg⁻¹ by nick-translation²⁰. The blot was hybridized for 36 h at 65 °C with 10⁶ d.p.m. cm⁻² of the denatured radioactive probe in 100 µl cm⁻² of hybridization solution: 0.9 M NaCl, 0.09 M Na-citrate, pH 7, 10% dextran sulphate, 0.5% SDS, 100 µg ml⁻¹ denatured herring sperm DNA, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin. The blot was washed twice for 10 min each at room temperature in 0.3 M NaCl, 0.03 M Na-citrate, 0.5% SDS, and three times for 1 h each at 65 °C in 0.15 M NaCl, 0.015 M Na-citrate, 0.5% SDS. The blot was autoradiographed for 3 days on Kodak XAR film using an intensifying screen at -70 °C. Sizes of hybridizing fragments were determined by comparing electrophoretic mobilities with a *Hind*III digest of bacteriophage λ DNA. b, 2 µg of each cosmid DNA (prepared as described elsewhere²¹) were digested with *Bam*HI, fractionated on a 1.0% agarose gel and transferred to nitrocellulose. Hybridization and washing were performed as in a except that the probe was used at 5 × 10⁴ d.p.m. cm⁻².

haemolytically active complement⁹. There are two related loci in the mouse, located in the *S* region of the *H-2* complex. One encodes mouse *C4*, and the other, *Slp*, encodes 'sex-limited protein', a haemolytically inactive homologue of *C4* expressed only in males of certain strains^{10,11}. To determine whether the mouse 21-OH gene was linked to the *C4* or *Slp* genes, Southern blots were prepared from overlapping cosmid clones spanning the *S* region⁷ and hybridized with the pC21a probe (Fig. 1b). The probe hybridized with a 3.8-kb *Bam*HI fragment in cosmids E-7 and E-69 and a 3.7-kb *Bam*HI fragment in cosmid E-26, corresponding to the 3.7-3.8-kb *Bam*HI fragments seen in Southern blots of total genomic DNA (Fig. 1a). An 8.5-kb *Bam*HI fragment was detected in cosmid E-15 which contained

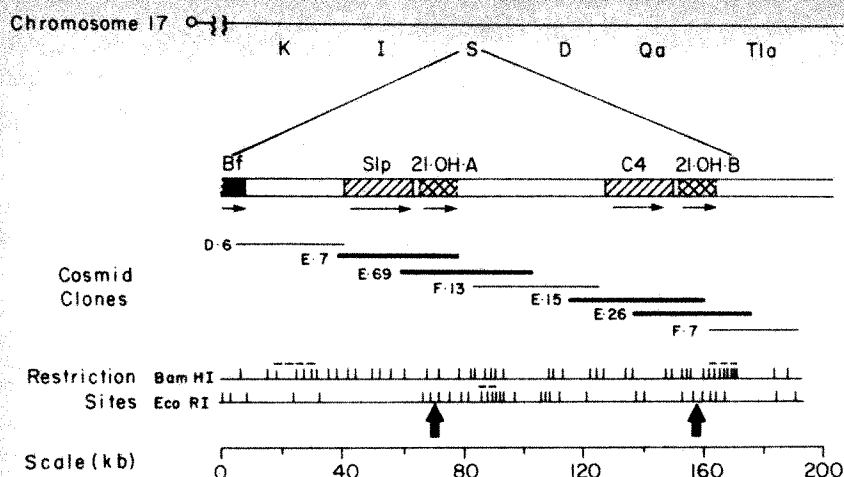


Fig. 2 Molecular map of the murine *S* region. The results of Fig. 1 are shown schematically. Cosmids⁷ carrying fragments which specifically hybridize with the pC21a probe are indicated by bold lines. Maps of *Bam*HI and *Eco*RI recognition sites are displayed above a scale in kb. A dotted line above restriction sites indicates that the order of fragments under the line has not been determined. The vertical arrows below the map indicate the fragments that hybridize to the pC21a probe. The proposed arrangement of the genes for factor B, *Slp*, *C4* and *21-OH* (*P-450_{C21}*) is illustrated at the top of the figure. Because the pC21a clone is not a full-length cDNA copy, the 5' and 3' borders of the two *21-OH* genes have not been determined and are therefore indicated by dotted lines. The direction of transcription is shown below each gene.

a portion of the 3.7-kb fragment seen in cosmid E-26 ligated to the cosmid vector, indicating that the sequence hybridizing with the probe was at one end of the genomic DNA insert. In addition, cosmids E-7, E-69 and E-26 all contained a 3.0-kb *Eco*RI fragment which hybridized with the probe and co-migrated with the *Eco*RI fragment identified in Southern blots of mouse genomic DNA (data not shown). In cosmid E-15, a smaller *Eco*RI fragment at the end of the insert hybridized with the probe.

Cosmids E-7 and E-15 respectively carry the *Slp* and *C4* genes^{7,12,13}. By inspecting the map of restriction sites for these clones (Fig. 2), it was possible to locate the sequences hybridizing with pC21a to fragments within 6 kb 3' of the *Slp* and *C4* genes. These experiments demonstrate that there are two *21-OH* genes in the mouse (which we have designated *21-OH-A* and *21-OH-B*) and that, as is true for at least one *21-OH* gene in man, they are tightly linked to the class III MHC gene, that for complement *C4*.

The adrenal cortex is the primary site of *21-OH* activity. To verify that the *C4*-linked *21-OH* genes were appropriately expressed in the mouse, the 3.8- and 3.7-kb *Bam*HI fragments from cosmids E-7 and E-26 were used to probe Northern blots of total RNA prepared from liver, testes and adrenal glands of C57BL/6 mice¹⁴ (Fig. 3). These probes hybridized to a 2.2-kb RNA species in total mouse adrenal RNA; this corresponds well to the size of *21-OH* mRNA isolated from bovine adrenal glands⁶. There was no apparent hybridization with unfractionated RNA from liver or testes, but very low levels of hybridization would not have been detected.

The orientations of the *21-OH* genes were determined relative to the *C4* and *Slp* genes. The 3.8-kb *Bam*HI fragment of cosmid E-7 (*21-OH-A*) was digested with *Kpn*I, yielding 2.7- and 1.1-kb subfragments. Restriction mapping demonstrated that the 2.7-kb fragment was closer to the 3' end of the *Slp* gene, whereas the 1.1-kb fragment was closer to the 5' end of the *C4* gene. The 1.1-kb *Bam*HI-*Kpn*I fragment was subcloned into the single-stranded bacteriophage vectors M13 mp8 and mp9 (ref. 15), and complementary single-stranded ³²P-labelled probes were prepared from each subclone¹⁶. The probe prepared from the mp8 subclone, but not the probe from the mp9 subclone, hybridized to a 2.2-kb species in total adrenal RNA (data not shown). Similar results were obtained with the 1.1-kb *Bam*HI-*Kpn*I fragment derived from the *21-OH-B* gene. These results demonstrate that both *21-OH* genes are transcribed in the same direction as the *Slp* and *C4* genes.

Previous study of these cosmid clones using several single-copy probes derived from regions flanking the *C4* and *Slp* genes⁷ indicated that the two genes arose by a direct duplication of about 55 kb of chromosomal DNA. The *21-OH* genes are located within this duplicated region; thus, single adjacent *C4* and *21-OH* genes were presumably duplicated simultaneously. The *Slp* gene product subsequently diverged from *C4* in function

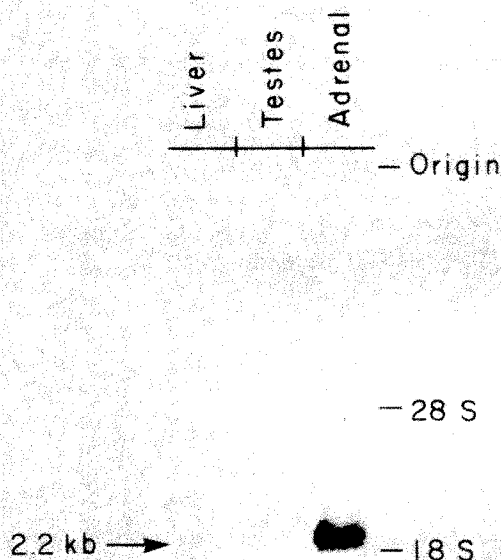


Fig. 3 Northern blot analysis using a murine genomic *21-OH* probe. Total RNA was isolated from the liver, testes and adrenal glands of C57BL/6 mice¹⁶. 15 µg of each RNA were fractionated by electrophoresis in denaturing conditions in 0.9% agarose-formaldehyde²² and transferred to nitrocellulose. The 3.8-kb *Bam*HI fragment of cosmid E-7 was nick-translated as described for Fig. 1a, and hybridized with the blot at 1×10^5 d.p.m. cm^{-2} in 0.1 ml cm^{-2} of: 50% formamide, 5 mM Tris-HCl pH 7.6, 0.75 M NaCl, 0.075 M Na-citrate, 12.5% dextran sulphate, 0.1% SDS, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1 mg ml^{-1} denatured herring sperm DNA. The blot was hybridized at 42 °C for 18 h, washed as described for Fig. 1a and autoradiographed for 4 h at -70 °C without an intensifying screen. Molecular sizes were estimated by comparison with 18S and 28S RNA, assuming sizes of 2,100 and 4,200 nucleotides, respectively. The same result was obtained when the 3.7-kb *Bam*HI fragment of cosmid E-26 was used as the probe.

and regulation; at present, it is not known whether both or one of the murine *21-OH* genes is active. There is also evidence for concerted regulation of the *21-OH* and *C4* genes, but the *21-OH* genes may indirectly influence expression of *Slp* by modulating the production of adrenal androgens¹.

Our mapping of the murine *21-OH* loci within the *S* region suggests that the *21-OH* genes became linked to the MHC before mammalian speciation. The functional significance of this linkage is unclear; it is possible that the *21-OH* genes have remained in the MHC because of their close proximity to the *C4* gene. Alternatively, as all other genes in this region have roles

immune function, it is possible that 21-OH exerts an immunomodulatory role by regulating glucocorticoid biosynthesis. There may also be non-immunological effects; inbred mice display mating preferences depending on H-2 type, and can discriminate urine odours from H-2 congenic mice in Y-maze experiments¹⁸. H-2-associated differences in 21-OH activity would be expected to influence urinary levels of metabolites of both glucocorticoids and androgens, which might be perceived by mice. Such an influence on mating preference might encourage an advantageous mixing of mouse populations. An analysis of the regulation of the two 21-OH genes in different inbred mouse strains may help to answer these questions.

We thank Ms Barbara Onufer and Mr Daniel New for technical assistance. P.C.W. is a Clinical Scholar of the Norman and Rosita Winston Foundation, D.D.C. is a recipient of a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research, and J.H.W. is supported by a fellowship from the American Cancer Society. This work was supported by NCI grants CA-22507 and CA-08748 (B.D.) and NIH grants HD-00072 and RR-47 (M.I.N.), and AI-18436 and AI 19148 (J.G.S.). *Note added in proof:* Mapping of human cosmid clones shows that in man, as in the mouse, there are two 21-OH genes which are located immediately 3' to each of the two C4 genes²³.

Received 6 June; accepted 20 September 1984.

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Correction of feline arylsulphatase B deficiency (mucopolysaccharidosis VI) by bone marrow transplantation

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Feline and human mucopolysaccharidosis VI (MPS VI or Maroteaux-Lamy syndrome) are inherited autosomal recessive deficiencies of lysosomal enzyme arylsulphatase B¹⁻⁶. Affected cats and children exhibit lesions caused by incompetent degradation, retinal atrophy and excessive urinary excretion of dermatan facial dysmorphism, corneal stromal opacities, leukocyte granulation, retinal atrophy and excessive urinary excretion of dermatan sulphate—and usually die before adulthood⁷⁻¹¹. Most attempts to

treat humans affected with MPS VI or other mucopolysaccharidoses have been ineffective or logistically prohibitive¹²⁻²¹, but allogeneic bone marrow transplantation (BMT) offers promise for cure of certain inborn errors of metabolism²²⁻²⁴. Engraftment of normal donor marrow may endow the enzyme-deficient recipient with a continuous source of enzyme-competent blood cells and tissue macrophages to facilitate degradation of stored substrate and to prevent genesis of further malformations. To test this hypothesis, we performed allogeneic BMT in a 2-year-old male Siamese cat with advanced MPS VI. Here we describe BMT-induced correction of this hereditary enzyme deficiency.

The MPS VI-affected 2-year-old male Siamese cat used in this experiment was the offspring of two known MPS VI heterozygotes⁷. A healthy female sibling with normal levels of arylsulphatase B activity was selected as marrow donor after it was found to be histocompatible with the affected cat on the basis of one and two-way mixed leukocyte nonreactivity—the sole criterion available for determination of feline histocompatibility (for methods see Fig. 1). Total-body irradiation was delivered to the affected recipient cat as a single 7.0-Gy dose at a rate of 2.5 Gy min⁻¹ with a 6-MeV linear accelerator after selective decontamination of its intestinal tract with oral neomycin and polymyxin B for 7 days. At 24 h post-irradiation, female sibling donor bone marrow cells (2 × 10⁸ per kg body weight) were injected into the jugular vein of the irradiated MPS VI-affected cat (see Fig. 1). For 40 days thereafter, the cat was housed in a laminar flow sterile isolator where it received sterile food and water and its temperature, hydration, and fluid intake and output were monitored daily. Parenteral fluids and antibiotics were given as indicated by a deficit in hydration status and rectal body temperature greater than 39.2°C, respectively. Blood leukocyte and platelet concentrations, volume of packed red cells, leukocyte arylsulphatase B activity, and urinary glycosaminoglycan (GAG) excretion were determined before and after BMT (for methods see Figs 1, 2). Cyclosporin (15 mg per kg orally) was administered daily from 19 to 104 days post-BMT to prevent graft-versus-host disease. Karyotypic analysis of cultured bone marrow cells was performed 183 days after BMT.

Irradiation-induced myelotoxicity was evident on day 1 and extended to day 12 after BMT (Fig. 1). Beginning 18 days after BMT, rapid reconstitution by donor-origin granulocytes, monocytes and thrombocytes occurred. An early indication of donor-origin engraftment was a marked decrease in Alder-Reilly body-bearing neutrophils, and appearance of Barr body-bearing (female) neutrophils. Lymphocytes remained below pretransplant levels during cyclosporin therapy (days 19 to 104) but returned to normal levels thereafter. Urinary dermatan sulphate excretion decreased 2.5-fold by day 1 and 14.6-fold by day 232 after BMT (Fig. 2). Leukocyte arylsulphatase B activity increased 30-fold by 232 days after BMT. Both urinary dermatan sulphate excretion and leukocyte arylsulphatase B activity have maintained values within the normal range. Karyotypic analysis on day 183 post-BMT revealed a stable chimaera of 72.5% donor-origin (female) and 27.5% recipient-origin (male) cells.

Clinical changes in the MPS VI-affected cat following restoration of arylsulphatase B activity by BMT have been complete resolution of corneal clouding and continuing resolution of facial dysmorphism (Fig. 3). Subjective changes in the cat after BMT include improved ability to walk and increased movement of the head, neck and mandible (for example, restored capacity to masticate dry food), increased suppleness of the haircoat and improved demeanour.

While the logic behind treating heritable disorders of bone marrow-derived cells with allogeneic BMT is evident (reviewed in ref. 23), the rationale for treating multisystemic lysosomal storage diseases, such as MPS VI, with allogeneic BMT is less clear. Implicit in the application of BMT therapy for conditions in which the biochemical defect is expressed in organs not derived from bone marrow, is the belief that either the adopted enzyme-replete cells will transfer enzyme to cells of the several involved organs²⁵⁻²⁸, or that bone marrow-derived cells are

Fig. 1 Peripheral blood cell numbers in the MPS VI-affected cat marrow transplanted with normal allogeneic feline bone marrow. Total-body irradiation-induced myelotoxicity (days 0-18) followed by bone marrow transplantation-mediated haematological reconstitution (days 18-25) and stabilization (days 25-250). **Methods:** At the times indicated, complete blood counts and serum chemistry evaluations were performed. Ficoll-metrizoate (1.077 g cm^{-3})-separated leukocytes from each of four siblings of the MPS VI-affected cat were assayed for one and two-way mixed leukocyte reactivity against similarly separated lymphocytes of the affected cat according to a modification of Peck and Bach³⁶. Lymphocytes (2×10^5) were mixed in 96-well plates either with 2×10^5 mitomycin C-treated ($2 \mu\text{g}$ mitomycin C per 10^6 lymphocytes, 45 min incubation at 37°C) or with 2×10^5 non-mitomycin C-treated leukocytes and incubated in a 37°C , humidified, 5% CO_2 atmosphere for 7 days. Eighteen hours before collection, $0.5 \mu\text{Ci}$ of ^3H -thymidine was added to each well. The cat with the lowest stimulation index was selected as the marrow donor. Marrow was obtained from the ketamine-anaesthetized donor by aspiration from both femurs and humeri. The cells were diluted in Hank's balanced salt solution, centrifuged at $1,000g$ for 10 min, the surface fat layer was removed, and the buffy coat cells were separated by pipette aspiration and then filtered through six layers of sterile gauze. Viable mononuclear cells were enumerated with the aid of Trypan blue vital stain and 2×10^8 mononuclear cells per kg infused into the jugular vein. Following haematological reconstitution (day 21) cyclosporin (a gift from J. F. Borel, Sandoz) was administered orally, $15 \text{ mg per kg daily}$. Serum trough levels of cyclosporin were measured at $134 \mu\text{g l}^{-1}$ (University of Minnesota Hospitals, Outreach Program). Karyotypic analysis was performed as follows: aspirated bone marrow mononuclear cells from the affected cat were cultured for 4 days in 25 cm^2 flasks as described³⁷, medium containing nonadherent cells were transferred to a 15-ml conical centrifuge tube, incubated for 1 h at 37°C with $4 \mu\text{g}$ of colchicine, and the pelleted cells were exposed to 5 ml of hypotonic (0.075 M) KCl solution for 10 min at 37°C and fixed with 1 ml of methanol:acetic acid (1:3). The final cell suspension was dropped on cleaned glass slides and dried at 65°C , and stained with a 2% Giemsa stain for 6-8 min. Forty metaphase chromosome were examined at $\times 1,000$.

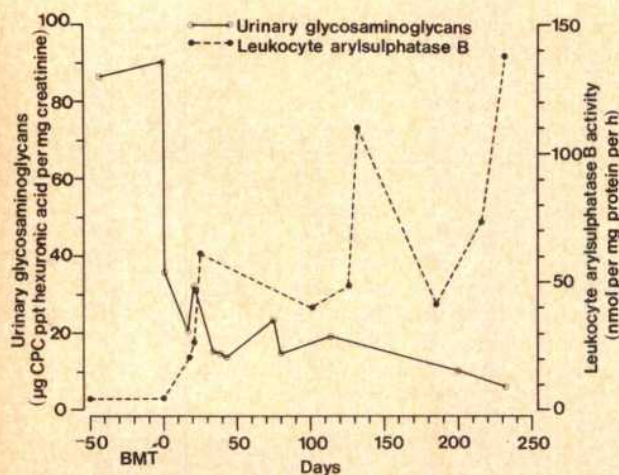
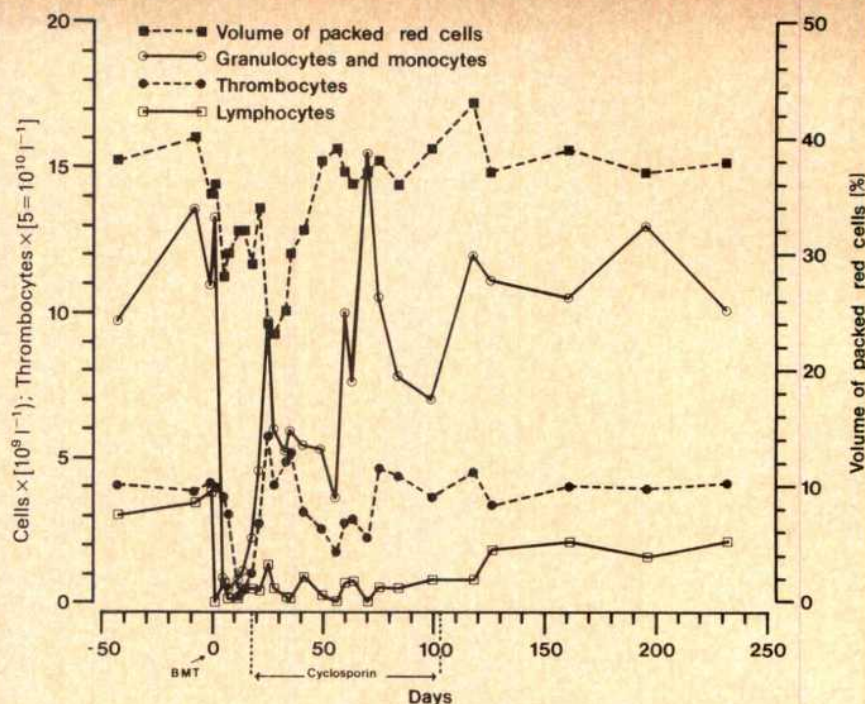


Fig. 2 Urinary excretion of glycosaminoglycans and leukocyte arylsulphatase B activity of the MPS VI-affected cat marrow transplanted with normal feline bone marrow. Urinary excretion of glycosaminoglycans decreased to the normal range (day +18 to day +232) as arylsulphatase B activity was installed in the mucopolysaccharidosis VI-affected cat by bone marrow transplantation. Urinary glycosaminoglycans were determined according to a modification of Pennock³⁸. CPC ppt, cetylpyridinium chloride precipitate. The predominant glycosaminoglycan excreted pre-BMT was dermatan sulphate, as determined by thin-layer chromatography. Leukocyte arylsulphatase B activity was determined according to a modification of Baum *et al.*³⁹.

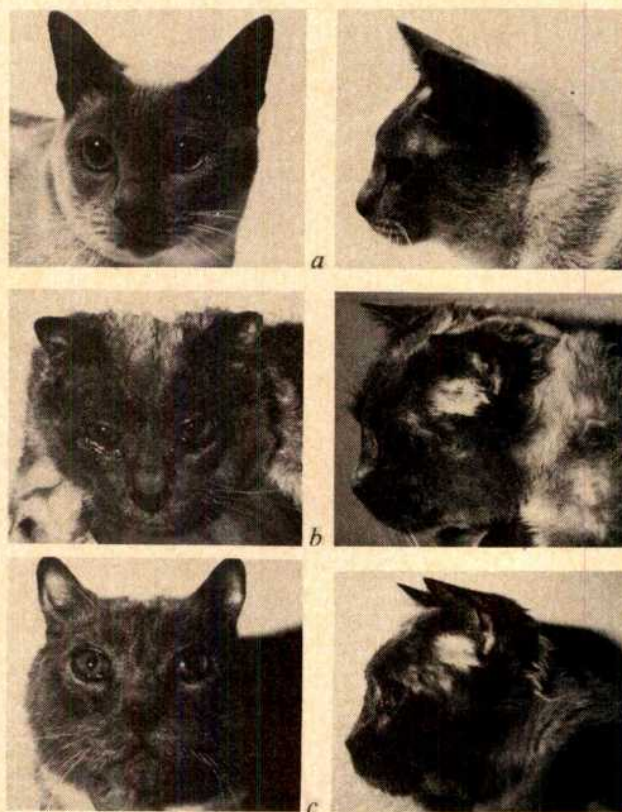


Fig. 3 a, Normal Siamese cat; b, arylsulphatase B-deficient sibling of the cat shown in a; c, the same animal 105 days after bone marrow transplantation. Complete resolution of corneal clouding and partial resolution of facial disfigurement can be seen.

responsible for degradation of the majority of systemic substrate under normal conditions. Support for one or both of these mechanisms is provided by the demonstrated correction of the galactosylceramidase deficit in the nerves of 'twitcher mice' when these nerves are grafted into normal mice²⁹.

Although allogeneic BMT has been performed in children with various lysosomal storage diseases, the long-term effectiveness of this treatment is unknown³⁰⁻³⁵. Our results show significant and sustained improvement in the health of an MPS VI-affected cat after successful allogeneic BMT, which suggests that allogeneic BMT may provide curative therapy for human MPS VI. However, in those lysosomal storage diseases where significant neurological damage occurs, this method must be further evaluated before it can be conscientiously recommended.

Received 19 June; accepted 6 September 1984.

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Investigations using this animal model of human disease could clarify the pathogenesis of MPS-induced lesions and would allow detailed analysis of the ability of BMT to prevent and correct pathological changes caused by arylsulphatase B deficiency and perhaps other inborn enzyme deficiencies.

We thank K. L. Martin for clinical monitoring of the cat, J. C. DeMartini, R. E. Smith and M. G. Weiser for review of the manuscript, and H. J. Deeg and W. C. Ladiges of the Fred Hutchinson Cancer Research Center, Seattle, for advice on transplantation techniques. This work was supported in part by grants F32-CA-06907, CA-32563, CA-32552, HD-08315, AM-33170, CA-29582 and by a grant from the Office of Development, College of Veterinary Medicine and Biomedical Sciences, Colorado State University.

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Unusual abundance of vertebrate 3-phosphate dehydrogenase pseudogenes

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Only one gene coding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), a key enzyme in the control of glycolysis, is known to be functional in man, mouse, rat and chicken¹⁻⁶. The gene has been localized to chromosome 12 in human^{2,3} and chromosome 6 in mouse⁴. Only a single mRNA species has been found in chicken⁷⁻⁹ and rat¹⁰. However, analysis of genomic DNA blots of various species with a cloned GAPDH cDNA probe has revealed large differences in the level of reiteration, ranging from one to over 200 copies. On this basis, we have grouped these organisms into three classes according to the number of GAPDH-related sequences they contain; one class with a unique representation (chicken), another class of relatively low reiteration (10-30 copies in man, hare, guinea-pig and hamster) and a third class of high reiteration (>200 copies in mouse and rat). The third class represents the first reported occurrence of such an extreme number of pseudogenes related to an enzyme-coding gene and suggests that a dramatic amplification event took place between 15 and 25 million years ago.

As a preliminary to the screening of genomic libraries to obtain the gene, we probed Southern blots¹¹ of chicken and rat genomic DNA, respectively, with GAPDH cDNA clones from rat (pR GAPDH1)¹⁰ and chicken (pGPD1)⁷. In chicken DNA, we obtain a simple pattern of *Eco*RI (10 and 15 kilobases (kb)) and *Hind*III (8, 1.7 and 0.5 kb fragments) (Fig. 1). The *Hind*III pattern is identical with that observed by Kuo *et al.*¹² and the *Eco*RI pattern is also in good agreement with previous reports^{6,12}, despite the presence of a weaker additional band, probably reflecting some polymorphism at *Eco*RI sites. All these data support the existence of a single GAPDH sequence in the chicken genome. In contrast, however, Fig. 1 shows a large number of fragments homologous to GAPDH cDNA in the rat genome. This clone consists exclusively of coding sequences (spanning amino acids 261-324)¹⁰, so this hybridization pattern does not result from repetitive sequences in untranslated regions¹³. Moreover, the probe is so small as to make it extremely unlikely that the multiple bands would correspond to genomic fragments harbouring different exons. To eliminate the possibility that the pattern of multiple bands detected results from incomplete digestion of the genomic DNA, the same blots were rehybridized to a rat serum albumin cDNA clone, yielding a pattern similar to the one published for this gene¹⁴ (data not shown). The stringency of washing conditions (0.2 × SSC, 60 °C) suggests that these multiple genomic bands are highly homologous to GAPDH. Indeed, the same pattern was observed when filters were washed either at 25 °C in 2 × SSC or at 73 °C in 0.2 × SSC (not shown). This is therefore a clear indication for a highly multigenic family of related sequences in the rat genome. To rule out a preferential reiteration of only part of the GAPDH sequence as observed previously in the human myoglobin gene¹⁵, we rehybridized mouse and rat Southern blots with a *Pst*I-*Hin*FI fragment excised from a full-length rat cDNA clone. This probe is 229 nucleotides long, including 74 5' untranslated and 155 coding nucleotides; it gave rise to the same complex pattern of bands (not shown), indicating that these

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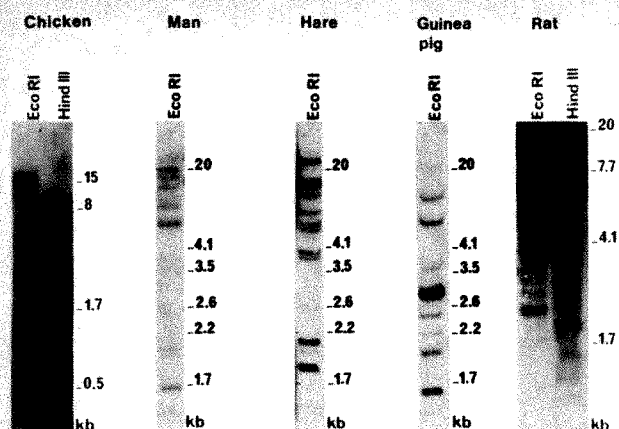


Fig. 1 Southern blot analysis of GAPDH-related sequences in chicken, hare, man, guinea pig and rat genomic DNA.

Methods: Genomic DNAs were prepared according to Gross-Bellard *et al.*²⁶. Hare DNA was a gift of Dr Y. Pauplin. Genomic DNAs (20 µg for chicken, man, guinea pig and hare, 5 µg for rat) were digested for 24 h with a 10-fold excess of the indicated restriction enzyme (Boehringer) and electrophoresed onto a 0.5% agarose gel (Sigma, type II) at 2 V cm⁻¹ for 20 h in Tris-borate-EDTA buffer (90 mM/90 mM/2 mM, pH 8.3). *EcoRI* and *HindIII* fragments of adenovirus 2 DNA were used as molecular weight markers (kilobases). Blotting onto nitrocellulose membranes was as described by Southern¹¹. Hybridizations were carried out in 50% formamide, 0.75 M NaCl, 50 mM sodium phosphate pH 7, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.5% SDS, 400 µg ml⁻¹ denatured sonicated salmon sperm DNA and 5–10 ng ml⁻¹ of nick-translated radioactive probe (1–2 × 10⁸ d.p.m. µg⁻¹, Amersham). The chicken blot was probed with a chicken cDNA clone (pGPD-1)⁷, the human, hare, guinea pig and rat blots with pRGAPDH-1 (ref. 9). Three 20 min washes in 2 × SSC (0.3 M NaCl, 0.03 M Na citrate) at room temperature were followed by a more stringent wash in 0.2 × SSC at 60 °C for 1 h. Autoradiography was performed at –80 °C on Kodak XAR5 film with an intensifying screen. Exposure times were 4 days for chicken, human, guinea pig and hare and 16 h for rat.

amplified sequences were not truncated significantly at the 5' end.

We next determined more accurately the number of sequences related to GAPDH cDNA and sought to determine when the amplification events occurred during evolution. Human, hare, guinea pig, hamster, mouse, chicken and rat DNA was analysed by Southern blot and dot-blot assay using a rat cDNA probe (pRGAPDH-1) (Fig. 2). The stringency of the washing conditions (0.2 × SSC, 60 °C) is such that only the least divergent sequences are scored, so that the values obtained are minimal estimates. We grouped the organisms into three classes according to the number of related sequences they contain. Class I has a single copy (for example chicken) and class II organisms have 10–30 copies (for example man, hare, guinea pig, hamster). The number of bands in Southern blots (Fig. 1) is consistent with the number of gene copies titrated by dot-blot assay (Fig. 2). The number of human GAPDH genes was confirmed by a homologous cDNA probe¹⁶. Class III (for example, rat, mouse) organisms contain an unusually high number of sequences (>200 copies), consistent with the results of screening a rat genomic DNA cosmid library with the pRGAPDH-1 probe, yielding an unexpectedly high number (140) of positive clones per ~0.8 genome equivalent (not shown), that is, approximately one clone per gene copy. To eliminate any possible artefact of the dot-blot assay, the same blot was probed successively with the rat serum albumin cDNA clone used above as a control of genomic blots and pRGAPDH-1. As expected, only one copy of albumin gene was titrated under these conditions (data not shown), confirming our finding of multiple copies of GAPDH-related sequences.

The high number of bands observed on all but chicken genomic blots (Fig. 1, hare and rat) argues against a clustering

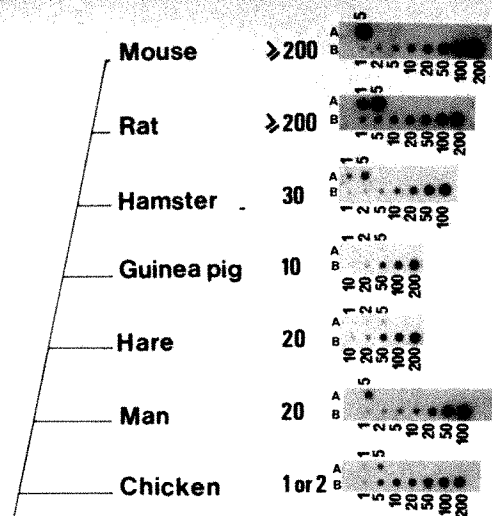


Fig. 2 Dot-blot quantitation of GAPDH-related sequences in various species. Lane A, indicated amounts (in µg) of denatured genomic DNAs probed with nick-translated chicken pGPD1 for chicken DNA and with pRGAPDH-1 for DNA from all other species. Lane B, scale of known amounts of pGPD1 or pRGAPDH-1 DNA 2 hybridized with the homologous probe in parallel with genomic DNA. The amount of plasmid DNA deposited on each spot of lane B was adjusted so that the numbers indicate directly the number of GAPDH sequences per 5 µg genomic DNA as spotted in A. The number of genes on the divergence tree are averages of independent experiments (5 for human, mouse, rat and hamster and 3 for chicken, hare and guinea pig). The probe used in all cases had comparable specific activities (1–1.5 × 10⁸ d.p.m. µg⁻¹). Samples of genomic and plasmid DNA were denatured by boiling for 10 min in 0.3 M NaOH at 50 µg ml⁻¹, chilled on ice and neutralized with one volume of 4 M ammonium acetate. Fixation onto nitrocellulose membranes used the BRL HybriDot system. Hybridization and washing were as described in Fig. 1.

of GAPDH-related sequences. At the chromosomal level, the presence of a processed gene on the human X chromosome^{17,18} indicates that these sequences are not confined to the human chromosome 12, the site of the only functional gene^{2,3}. At the level of rat genomic DNA, the rough equivalence between cosmid clones and gene copies mentioned above further indicates that there should not be more than one copy per ~40 kb.

The three classes of reiteration seem to have arisen through at least two steps of amplification which, according to the divergence tree of Fig. 2, could be dated back to more than 70–80 million years¹⁹ (after the separation of birds and mammals) and to 15–25 million years^{19,20} (after separation of Cricetidae and Muridae phyla but before that of species within the Muridae). However, the high degree of sequence conservation between rat and all other organisms except chicken argues against the first amplification step. Using a value of 5 × 10⁻⁹ nucleotide substitutions per site per year as a mutation rate for a non-functional gene²¹, one would expect at least 35% divergence between the true gene (represented by the cDNA probe) and pseudogene to have arisen before the separation of human from the other species analysed (70–80 million years). This is certainly not the case as, under the hybridization stringency used here, these pseudogenes would not be detected. Indeed, rat and chicken cDNA which have diverged by 19% (ref. 10) did not cross-hybridize under our stringency conditions.

The nature of the amplification processes is thus open to speculation. Duplications and random insertions of cDNA sequences (processed genes or retrogenes) in the genome are two mechanisms currently proposed for pseudogene generation. As mentioned above¹⁸, one such processed gene has been identified on human chromosome X, which indicates that random insertions can account for at least some of the GAPDH family.

In the case of human arginosuccinate synthetase, which shows approximately the same level of reiteration frequency (10–15 copies)²², it has been shown that most if not all are processed genes²³. Both GAPDH and arginosuccinate synthetase are ubiquitous housekeeping enzymes which are therefore likely to be expressed in germ-line cells where retrogenes are believed to be generated. Based on the above considerations, our hypothesis is that most, if not all, pseudogenes for these two enzymes could have arisen in man by insertion of cDNA copies. The same may also be true for the other members of the same class of reiteration (hare, guinea pig and hamster).

On the other hand, several considerations make it unlikely that the second mechanism alone could have produced such a remarkable multigenicity as observed in Muridae. First, insertion of cDNA copies in such a high number has never been observed, even for genes encoding other ubiquitous proteins such as actin²⁴ or dihydrofolate reductase²⁵ for which mRNA templates are available constantly. Second, a preliminary analysis of several genomic clones shows that different clones have slightly different patterns of stained bands but similar patterns of GAPDH-hybridizing bands, suggesting that duplication events involving DNA fragments of several kilobases in length occur in these organisms.

Our findings make it possible to speculate on the relative contribution of these two processes to the generation of the GAPDH family in Muridae. In a first step common to all species studied, except chicken, 10–30 pseudogenes would be contributed by insertion of cDNA copies. At this point, Muridae would have departed from other species by a burst of duplication events (possibly by transposition-like mechanisms) to reach the extreme degree of reiteration observed. A detailed analysis of our characterized rat genomic clones is now necessary to elucidate the mechanisms involved in this latter step.

During the completion of this work, we learned that A. Hanauer and J. L. Mandel (personal communication) have observed values quite similar to ours for the multigenicity of human, hamster and mouse.

This work was supported by grants from CNRS, INSERM, DGRST, CRAM, FNCLCC and Ligue Contre le Cancer. We thank Dr A. McLeod for providing the chicken pGPD1 clone, Drs P. Fort, G. Cathala and Y. Pauplin for helpful discussions and Drs J. L. Mandel and K. Davis for communicating their results prior to publication. We also thank Dr J. F. Julien for the rat cosmid library and Dr J. Marie for help in replication.

Received 24 July; accepted 13 September 1984.

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Intensification of the 5.9-nm actin layer line in contracting muscle

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According to the cross-bridge model^{1–3} of muscle contraction, an interaction of myosin heads with interdigitating actin filaments produces tension. Although X-ray equatorial diffraction patterns of active (contracting) muscle show that the heads are in the vicinity of the actin filaments, structural proof of actual attachment of heads to actin during contraction has been elusive⁴. We show here that during contraction of frog skeletal muscle, the 5.9-nm layer line arising from the genetic helix of actin⁵ is intensified by as much as 56% of the change which occurs when muscle enters rigor, using a two-dimensional X-ray detector. This provides strong structural evidence that myosin heads do in fact attach during contraction^{6,7}.

Two-dimensional detectors of multiwire type^{8–10}, despite their advantage over the one-dimensional detector, have not been applied to structural studies on muscle because the spatial resolution in the direction perpendicular to anode wires is limited. We have improved the resolution by the method¹¹ used in a wide-angle detector, that is by increasing the drift distance for electrons liberated on ionization. The improved detector, like other position-sensitive detectors, has the capability of fast recording and low background and is therefore suitable for studying transient changes of weak X-ray reflections such as arise from the actin filaments during contraction.

The basic structure of the detector was the same as that described previously^{12,13}. Anode wires were sandwiched between two planes consisting of cathode strips (Fig. 1). These strips were parallel to the anode wires in the back plane and orthogonal in the front plane. An avalanche occurring to an anode wire induced positive pulses on nearby strips on both planes. Such pulses were transmitted to delay lines^{8,14}. The x coordinate of the avalanche was determined from the signal propagation times to both ends of the delay line in the back plane, and the y coordinate from those in the front plane.

The detector had a sheet of aluminized Mylar with a strong negative bias near the entrance of the chamber. This enlarged the space for electrons to drift and multiply, increasing the number of anode wires involved in each avalanche. In consequence, the accuracy of the position determination along the x axis was improved considerably. With an optimum spacing (4 cm) between the Mylar and the anode, the spatial resolution (full width at half maximum) was 600 μm along the x axis and 500 μm along the y axis.

The outputs of the detector were sent to a buffer memory (2 × 10⁴ words) which stored them as a function of time. After a data-collection period, consisting of a resting phase before muscle contraction and an active phase during contraction, the contents of the buffer memory were transferred to a disk memory. The disk had separate memory segments for the two phases and accumulated the data on repetition of contractions to give rise to the diffraction patterns.

A sartorius muscle of the bullfrog *Rana catesbeiana* was placed in a low-angle camera of the mirror-monochromator type¹⁵ at a specimen-to-detector distance of 75 cm. The meridional and layer-line reflections at orders of 1/42.9 nm⁻¹, arising from myosin, were recorded up to the 6th order with a total exposure of 2–3 min. The exposure time needed for recording the much weaker actin layer line at 1/5.9 nm⁻¹ was 4–10 min, considerably shorter than the time (~24 h) required for

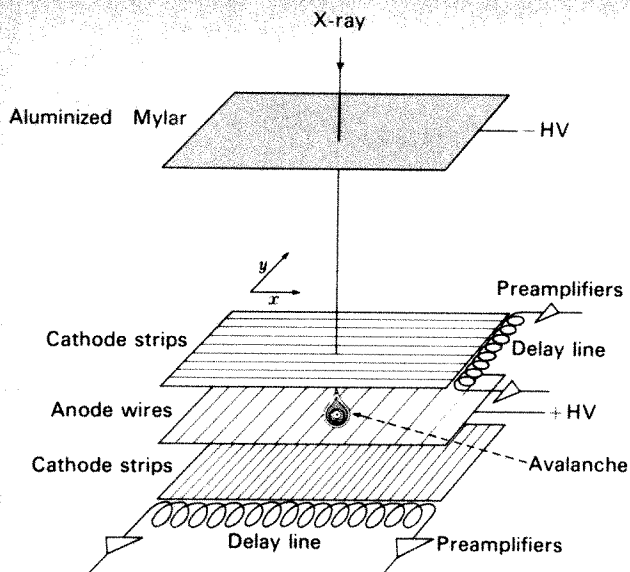


Fig. 1 Structure of the multiwire chamber. The chamber consisted of a sheet of aluminized Mylar (-1.0 kV) near the entrance, and an anode plane (80×80 mm, $+1.8$ kV) sandwiched by two cathode planes with a spacing of 3 mm. The anode plane was made of gold-plated tungsten wires $20 \mu\text{m}$ in diameter, 1.5 mm apart. The cathode planes were made of the same wires, 1 mm apart, orthogonal or parallel to the anode wires. The cathode wires were connected in pairs to form strips^{10,12}. The circuit for the delay-line readout was the same as that described by Hendricks¹³, containing four filter amplifiers, four discriminators and two time-to-digital converters. The sensitivity of the detector was not quite uniform along the x axis. When the uniformity was tested at a Mylar-to-anode distance of 4 cm with an uncollimated iron source, the photon count at each anode wire was 8% greater than that at the midpoint between wires. The chamber was filled with a gas mixture of 90% argon and 10% methane at a pressure of 1 atm. With a dead time of $7 \mu\text{s}$, the maximum counting rate was $4 \times 10^6 \text{ s}^{-1}$ with a 72% efficiency.

recording it on an X-ray film placed at the position of the detector.

We studied the change in intensity of this actin layer line on activation of muscles. Each muscle was stimulated to contract isometrically at a sarcomere length of $2.3 \mu\text{m}$ for 1 s every 5 min. The muscle produced a steady tetanic tension from about 0.15 s after the onset of the stimulus. The diffraction pattern obtained for a 0.8 s period during steady tension (Fig. 2a) was compared with the resting pattern (Fig. 2b) obtained for a 0.8 s period immediately before each contraction. During contraction, the integrated intensity of the actin layer line was $31 \pm 3\%$ ($\pm$ s.e.m., six pairs of patterns) greater than that during rest; the increase was statistically significant ($P < 0.01$). There was no apparent shift of the intensity peak along the layer line.

The layer line was intensified also in rigor muscles. The muscle was put into rigor while in the X-ray camera by first perfusing the specimen chamber (holding a living resting muscle at a sarcomere length of $2.3 \mu\text{m}$) with Ringer's solution containing 1 mM iodoacetate at 30°C for 1 h. The muscle then was stimulated repetitively with electrical pulses (1 Hz) until the resting tension increased and there was no response to stimulation. Finally the specimen chamber was perfused again with the iodoacetate Ringer for 1 h. In the rigor pattern the integrated intensity of the actin layer line was $55 \pm 7\%$ ($\pm$ s.e.m., five pairs of patterns) greater than that in the resting pattern. In addition, the radial position of the intensity peak was now closer to the meridian. Both the intensification of the layer line and the inward shift of the peak on rigor development had been shown previously¹⁵ and attributed to the attachment of myosin heads to actin.

When the behaviour of this layer line during contraction was

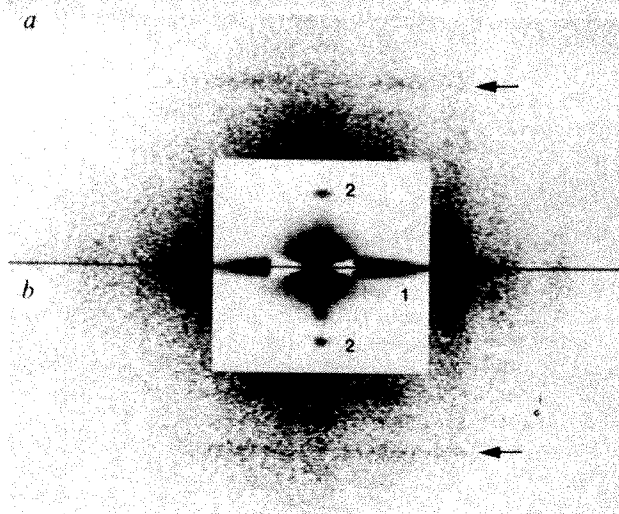


Fig. 2 Comparison of contracting (a) and resting diffraction (b) patterns. Arrows, actin layer lines at $1/5.9 \text{ nm}^{-1}$. In the central parts of the patterns, the contrast was adjusted to display the myosin layer line at $1/42.9 \text{ nm}^{-1}$ (1) and the meridional reflection at $1/14.3 \text{ nm}^{-1}$ (2).

Methods: A frog sartorius muscle was held in a specimen chamber by clamping the pubic bone at one end and connecting the tendon to a force transducer at the other end. The muscle axis was aligned parallel to the y axis of the detector. The specimen chamber was perfused with oxygenated Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl_2 , 2.15 mM Na_2HPO_4 , 0.85 mM NaH_2PO_4 , $\text{pH } 7.2$) at 2°C . Isometric contractions of 1 s duration were elicited by trains of supramaximal pulses (20 Hz) given through a pair of platinum electrodes placed parallel to the muscle axis. Each muscle was made to contract 40 times, and the diffraction data from 20 muscles were accumulated to obtain the contracting pattern; the total exposure time was ~ 10 min. The average tension of the 40 contractions was $84 \pm 1\%$ ($\pm$ s.e.m., $n = 20$) of the initial tension. The X-ray source was a rotating-anode generator (Rigaku, type FR) with a fine focus (1×0.1 mm) on a copper target. This was operated at 50 kV with a tube current of 70 mA.

first studied using X-ray films^{15,16}, no change in the intensity was detected. Haselgrove¹⁷ did observe an intensity increase during contraction but, because of the large standard deviations of the measurements, he considered this result inconclusive. A one-dimensional position-sensitive detector revealed no intensity change¹⁸. The present study has demonstrated a significant intensity increase, more than 50% of that occurring on rigor induction. Dr H. E. Huxley (personal communication) and his co-workers have observed similar changes.

Other possible causes of an intensity increase of this layer line during contraction have been eliminated by Parry and Squire⁶, who concluded that the increase can be explained satisfactorily only in terms of the attachment of myosin heads to actin. Thus our result provides evidence for the attachment of cross-bridges in active muscles⁷, although at this stage we cannot rule out the possibility that other changes in the thin filament contribute to the increase. The absence of significant inward shift of the intensity peak along the layer line can be explained by assuming the attachment to occur at a variety of angles^{6,7}.

We thank Professors S. Ebashi and M. Endo for advice, and Drs E. J. O'Brien, G. W. Offer and D. W. Maughan for discussion. This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture, Japan.

Received 27 July; accepted 2 October 1984.

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A sequence downstream of AAUAAA is required for rabbit β -globin mRNA 3'-end formation

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The sequence AAUAAA, found 11-30 base pairs (bp) upstream of the poly(A) site of most non-histone eukaryotic messenger RNAs (mRNAs)¹ forms an essential part of the recognition site for 3'-end processing of the primary transcript²⁻⁴. However, the sequence AATAAA is found in transcribed regions of genes⁵⁻⁸ and is differentially utilized in genes containing multiple copies of the sequence within the 3'-noncoding region⁹⁻¹⁸, suggesting that the hexanucleotide alone does not comprise a complete recognition site. Therefore, it seems likely that additional sequences are required to form a complete recognition site for 3'-end formation. We have investigated the sequence requirements for mRNA 3'-end formation using the rabbit β -globin gene as a model system. Here we demonstrate that an additional sequence 3' to AAUAAA is required for the correct 3'-end formation of rabbit β -globin mRNA.

To delineate possible sequence requirements for 3'-end formation within the 3'-flanking region of the rabbit β -globin gene, *Bal*31 deletions were made of sequences downstream to AATAAA (Fig. 1). A simian virus 40 (SV40)-pBR328 expression vector, pB5'SV, containing the rabbit β -globin gene plus 425 bp of 5'- and 355 bp of 3'-flanking sequence¹⁹, was linearized at a unique *Sal*I restriction enzyme site 3' distal to the AATAAA and the ends digested with *Bal*31 exonuclease. This approach generated a series of clones with varying lengths of 3'-flanking deletions proceeding from a distal to proximal direction with respect to the AATAAA. A *Bgl*II fragment containing the 3'-noncoding region, the AATAAA plus the remaining 3'-flanking sequence was subcloned into the unique *Bgl*II site at the translation termination codon of the rabbit β -globin gene in the intact pB5'SV plasmid; this provided cloning sites 5' and 3' to the insert containing the *Bal*31 deletion, ensured that sequences adjacent to the insert were identical between constructs, and also retained the original 3'-noncoding region, AATAAA and poly(A) site 3' to the insert as an internal control.

The DNAs from these deletion constructs were transiently expressed in HeLa cells²⁰; 36 h after transfection, the cytoplasmic RNA was extracted and analysed by S₁ nuclease mapping^{21,22} using an end-labelled probe spanning the entire region of the insert and the original 3'-flanking region downstream. RNAs from constructs showing correct 3'-end formation at the inserted site were poly(A)-selected (data not shown)²³.

Six constructs (clones 2-7) containing varying lengths of 3'-flanking deletions were analysed (Fig. 2). Clone 2, containing the largest 3'-flanking deletion ending 15 bp beyond the AATAAA, did not contain sufficient sequences to signal 3'-end

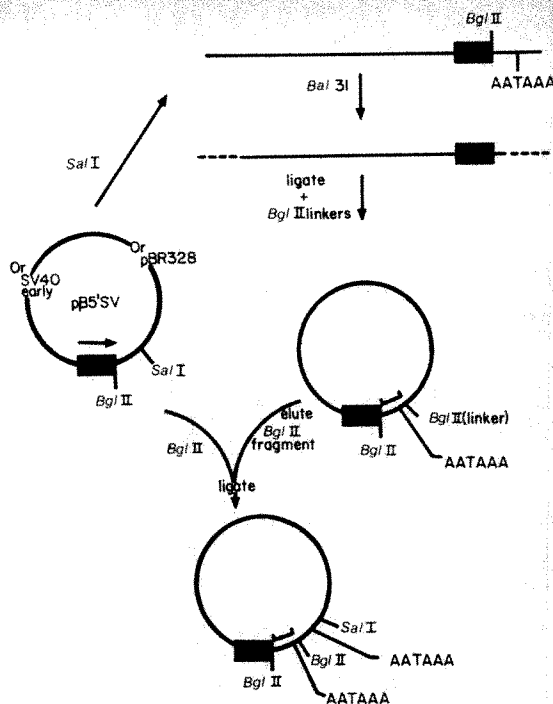


Fig. 1 Construction of *Bal*31 deletions 3' distal to the AATAAA sequence. The plasmid pB5'SV containing the entire rabbit β -globin gene plus 425 bp of 5'- and 355 bp of 3'-flanking sequence was linearized with *Sal*I at a unique restriction enzyme site 3' distal to the AATAAA. Linearized DNA (100 ng) was digested with 0.1 U of *Bal*31 exonuclease in a total volume of 10 μ l at 30 °C for 35-40 min²⁷. Approximately 20 ng of this DNA were filled-in and the ends ligated in the presence of an equal weight of unphosphorylated *Bgl*II linkers. The ligated DNA was transformed into MC1061 competent cells. Ampicillin-resistant transformants were selected and the DNA from mini-preparations²⁷ was digested with *Bgl*II. The *Bgl*II fragment containing the 3'-noncoding region, AATAAA, plus the remaining 3'-flanking sequence was eluted from a 1% agarose gel²⁷. This fragment was subcloned into the unique *Bgl*II site at the translation termination codon in the rabbit β -globin gene of the intact pB5'SV plasmid. The final constructs contained the insert flanked by *Bgl*II sites and the original 3'-noncoding region, AATAAA, and poly(A) site 3' to the insert.

formation within the inserted fragment. Instead, processing occurred 3' beyond, using the original AATAAA and poly(A) site (Fig. 3). The remaining five deletion constructs (clones 3-7) contained sufficient sequences to signal correct 3'-end formation 19 bp downstream of the inserted AATAAA. Of these five functional constructs, clone 3 contained the largest deletion of 3'-flanking sequence, ending 51 bp beyond the AATAAA, leaving a difference of only 35 bp between the end of this clone and the nonfunctional construct, clone 2. Within this 35-bp region is a G+T-rich homology, part of a pentanucleotide homology, and the poly(A) site. The G+T-rich homology, TGTGTTGGA, is found 3' of the poly(A) site in several other genes²⁴. The 5' end of this critical 35-bp region intersects another sequence homology, CAYUG, which is found 3' of the AATAAA before or after the poly(A) site of many vertebrate genes²⁵. This pentanucleotide sequence is complementary to regions of the RNA of the U4 small nuclear ribonucleoprotein, suggesting that U4 may mediate 3'-end processing. The poly(A) site is found 19 bp 3' of the AATAAA and is therefore missing from the nonfunctional deletion clone 2. However, sequence analysis of many vertebrate genes has shown that all four nucleotides are used for polyadenylation (S. M. Berget, unpublished data), thus the exact sequence of the poly(A) site is unlikely to be important for mRNA 3'-end formation. It is more likely that either or both of the G+T-rich and pentanucleotide sequence homologies may

Fig. 2 Sequence 3' to AATAAA, indicating the ends of the *Bal*31 deletion constructs, homologies found within the critical 35-bp region between the ends of the nonfunctional (2) and functional (3) clones, and the poly(A) site(*). The ends of the *Bal*31 deletion clones 2 and 3 were determined precisely by DNA sequencing²⁸ while those of clones 4–7 were determined approximately by restriction enzyme mapping. The numbering system corresponds to that used in Fig. 3.

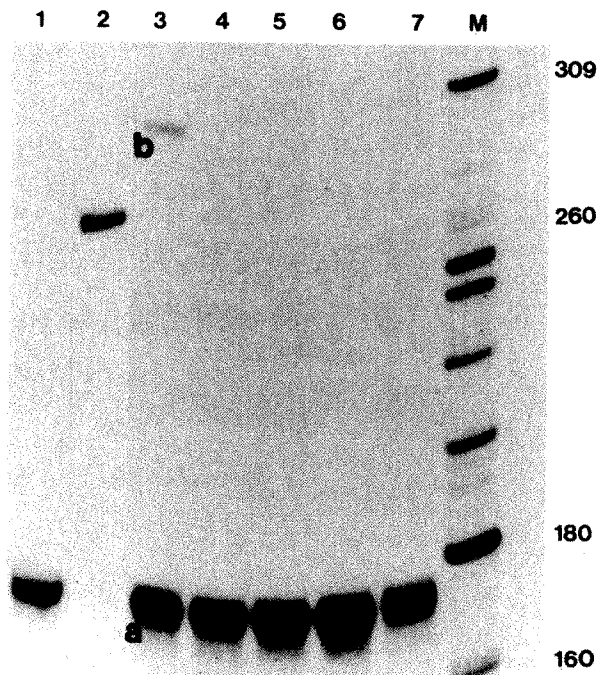
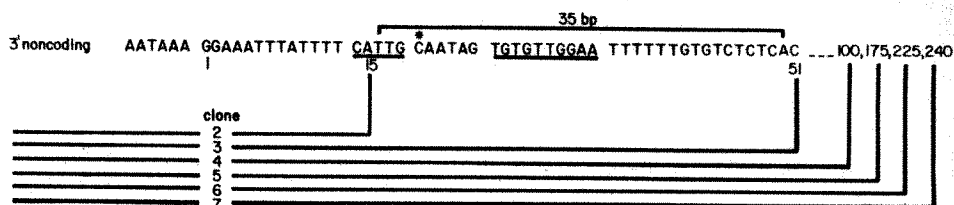


Fig. 3 S_1 nuclease mapping of the 3' ends of the cytoplasmic RNAs from *Bal*31 deletion constructs of the rabbit β -globin gene. Lane 1, (control) RNA from intact rabbit β -globin gene in the p β 5SV plasmid showing correct 3'-end formation 19 bp 3' of the AATAAA. Lane 2, RNA from the nonfunctional deletion construct, clone 2, with insert ending 15 bp beyond the AATAAA; 3' ends were formed only at the original poly(A) site 3' to the inserted fragment. Lanes 3–7, RNAs from functional deletion constructs with inserts containing progressively greater lengths of 3'-flanking sequence. Clones are arranged in ascending order, starting with clone 3, which ends 51 bp 3' of the AATAAA. a, Correct 3'-end formation occurred in all five clones 19 bp downstream of the inserted AATAAA. b, Some 3' ends, not detected in clones 4–7, were formed at the original poly(A) site 3' of the insert in clone 3. Lane M, ^{32} P-labelled markers (bases). The numbering system corresponds to that used in Fig. 2.

Methods: 10 μ g of DNA from each deletion construct were transfected into a half-confluent 199-mm dish of HeLa cells by the calcium phosphate precipitation method²⁰. Five plates were used for each DNA; 12 h after transfection the medium was changed. The cells were grown for an additional 24 h, then the RNA was extracted and the cytoplasmic fraction purified²⁷. The rabbit β -globin RNAs were detected by S_1 nuclease mapping of the 3' ends²² using an end-labelled RI-*Sal* DNA probe spanning the region of the insert and the original 3'-noncoding region, AATAAA and 3'-flanking sequence.

be involved in the formation of a complete recognition site for 3'-end formation of the rabbit β -globin mRNA as the nonfunctional deletion clone 2 ends 15 bp beyond the AATAAA and lacks both of these sequences.

Aside from correct 3'-end formation at the inserted site, a low level of processing, not observed in clones 4–7, was detected at

the original poly(A) site 3' to the inserted site in clone 3 (Fig. 3). If the low level of 3'-end formation at the original site accurately measures the amount of readthrough RNA not processed at the inserted site, then there might be sequences 3' to the poly(A) site that are not required for correct 3'-end formation but nevertheless affect the efficiency of the process. Alternatively, there may be a transcription termination sequence within the immediate 3'-flanking region, resulting in less readthrough RNA processed at the original poly(A) site.

The experiments described here demonstrate that sequences within a critical 35-bp region extending from 3 nucleotides 5' to 31 nucleotides 3' of the poly(A) site are required for correct 3'-end formation of the rabbit β -globin mRNA. These results are consistent with the *Bal*31 deletion analysis of the adenovirus E2A transcriptional unit recently described by McDevitt *et al.*²⁶; they demonstrated a sequence requirement for 3'-end processing downstream of the poly(A) site. However, the sequence of the required region of the E2A poly(A) site differs from that of the 35-bp region of the rabbit β -globin gene; this disparity may reflect the different factors involved in recognizing a signal sequence for 3'-end formation in a viral as opposed to a mammalian gene. As the functional clone ending 51 bp beyond the AATAAA includes the G+T-rich homology and the pentanucleotide homology, either or both of these sequences may be required for correct 3'-end formation. Further analysis by point mutation of the 35-bp region should elucidate the precise sequence requirement for 3'-end formation.

We thank F. Grosveld for plasmid p β 5SV, and D. Anson and I. Jones for helpful comments on the manuscript. A. G. and N. J. P. were supported by MRC project grant no. G8108316CB.

Received 28 August; accepted 7 October 1984.

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Lines of investigation

J.T. Bonner

Scaling: Why is Animal Size so Important?

By Knut Schmidt-Nielsen.

Cambridge University Press: 1984. Pp.241. Hbk £20, \$29.95; pbk £7.95, \$9.95.

Size, Function, and Life History.

By William A. Calder III.

Harvard University Press: 1984. Pp.431. \$32.50, £26.

THERE has been a rash of interest recently in the ramifications of the effect of size on living organisms. Traditionally this is a subject that has been within the province of vertebrate physiologists, although it is now extending, with important messages, into the realm of ecology.

Professor Schmidt-Nielsen's *Scaling* is written for a general audience and is eminently readable; a splendid book primarily on the effect of animal size on physiology. Schmidt-Nielsen has himself done important work in the field and this has been one of his interests for many years. His latest account of the subject is clearly written in much the same easy-to-follow way that characterizes *Animal Physiology* (Cambridge University Press, 3rd Edn 1984), his well-known textbook. Professor Calder's book, on the other hand, is for the specialist. It covers some of the material on physiology that is in Schmidt-Nielsen, but goes on to look into the developmental and ecological consequences of size. This is a very difficult book to read, yet it is full of valuable, detailed information, well worth any trouble it might cause the reader in digging out the nuggets. It is more of a reference compendium than a book to read, but there are short portions scattered here and there that suddenly flash out as important and useful insights. When I finished reading these volumes I felt they could well have been glued together, with Schmidt-Nielsen first as a lucid introduction, followed by Calder with fascinating detail. Since this is not possible, I urge the unwary to read them in that sequence. The appropriateness of this is not entirely without cause for one gathers from Calder's preface that he is a student and a friend of Schmidt-Nielsen.

Both books begin with the idea that an allometric formulation is the most useful way to compare size-connected or size-invariant properties of different organisms. This method dates back at least 50 years, to the work of Julian Huxley who compared the growth rates of two developing structures in such a way that they produced straight lines when plotted logarithmically. The slope of the line reflects the change of proportions with absolute size increase. This same method can apply equally well to any measurement and its relation to body size, and is the way in which these two authors most frequently use their allometry. So, for instance, to give

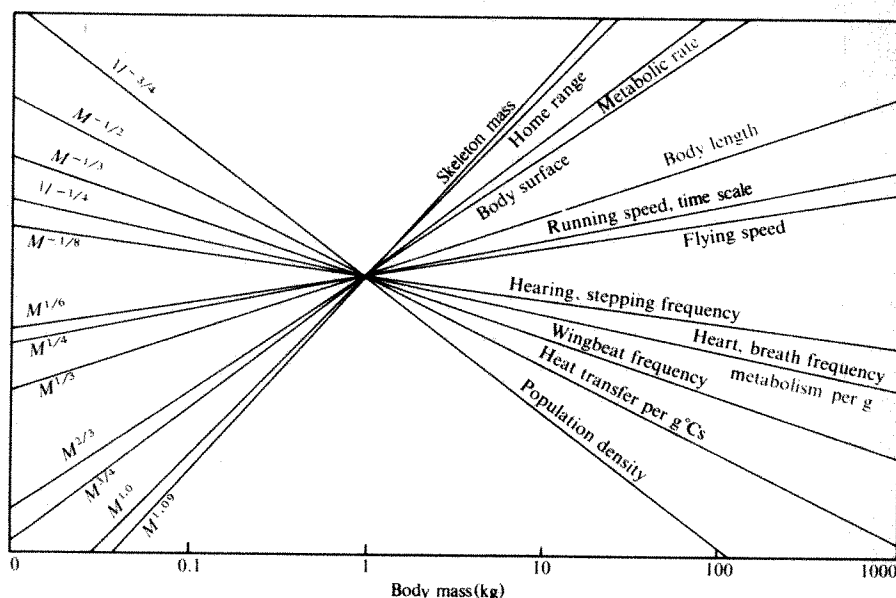
the celebrated example of M. Kleiber (and others), if metabolism is determined for different-sized organisms, then metabolism $\propto$ weight^b

where, in this case, $b = 3/4$. By substituting other components for metabolism one can conveniently express how they change with weight. One could substitute heart rate, or gestation period, or speed of locomotion, or brain size, or clutch size, or life span, or population density or the size of the home range, for they all vary with the body size of the animal.

The diverse properties which are described in this way can be roughly grouped into the following three categories: physiological (or functional), developmental and ecological. Only Calder discusses the last two. On the importance of body size in physiology there is now a vast array of new data. For example, it is possible to show that even though different organs in the body of a mammal may differ in their metabolic rates, all together in the whole organism they maintain the weight to the $3/4$ power relation, and this is re-

flected in the relative amounts of cytochrome c in the animal, as well as the cytochrome oxidase content, and even the relative number of mitochondria per cell for those tissues that have been examined. While this particular example is from Schmidt-Nielsen, Calder has many more where one can see complex relations between various structures, either in their relative size or the relative rates at which they function.

These allometric properties are often interconnected so that one can be derived from another. This raises the big question: is there one principle that underlies the connections between body size and body proportions, rates of locomotion and metabolism? One original and bold insight is that of T.A. McMahon in his well-known paper (*Science* 179, 1201-1204; 1973) where he suggested that different-sized organisms may keep their elastic properties similar; and once this is achieved one can, on theoretical grounds (too lengthy to describe here), make predictions that accord well with the shapes of trees and bones, and the $3/4$ rule for metabolism, and the allometric relations measured for quadruped locomotion. This elastic similarity model is discussed by both authors and they also carefully present the empirical evidence that seems to be at variance with it. Time will tell to what extent the McMahon hypothesis prevails, but at the moment it is the most comprehensive, all-embracing explanation of this family of interconnecting allometric relations. One might assume that natural selection favours similarity of elastic properties to avoid buckling in



Some allometric generalizations, mostly for eutherian mammals. When plotted on log-log paper, the fractional exponential powers of body mass appear as the slopes of the lines. For example, body surfaces of animals, like surface areas of geometric figures, are proportional to volume (or mass, assuming constant density) raised to the $2/3$ power. On the log-log plot the $2/3$ becomes the slope, that is, an increase of 10^2 in surface when mass increases by a factor of 10^3 . (Re-drawn from a figure in *Size, Function, and Life History* (p.11), after several authors.)

larger organisms, and this could conceivably be the cause of various functional and structural size-related properties. But the question of which properties are affected by selection and which are the inevitable consequences of the internal engineering of an organism remains a difficult one.

Two points stem from the above. One is Calder's important caution that allometric interrelations are statistical correlations and they therefore cannot tell us anything about causes. The other is a point made by both authors: it may not always be the examples that come close to the allometric mean that are interesting, but the exceptions. For instance, Scholander and his colleagues showed that an arctic weasel has an exceptionally high metabolic rate, well above the norm for its size; and they suggested that because it is small, and has short legs, its fur could not be thick enough to keep it warm in winter. The weasel's solution to the problem is to stoke its internal fires to a higher rate. Another interesting case is the hearts of small shrews and hummingbirds which are larger than the expected mean. The suggestion is that were this not so their hearts would have to beat at a physically impossible rate in order to supply sufficient blood flow; they compensate by increasing their heart to a size that has a lower, but mechanically possible natural frequency. Both these examples show that selection can, no doubt within limits, alter one feature to the exclusion of the others.

The use of allometry in ecology is a new

and exciting subject, but still in its infancy. It has also been reviewed by R.H. Peters in his recent book (*The Ecological Implications of Size*, published by Cambridge University Press in 1983) in a somewhat different way from Calder, although the two books overlap to some degree. What Calder does, which is especially useful, is to consider reproduction and development, as well as population properties such as animal density and home range size, for they are all interrelated. Often information about one of these subjects provides important insights into others. This is shown particularly well in the very recent work of P.H. Harvey, R.D. Martin and others on brain size and its ecological implications, work that is in progress and therefore not reported in any of these books. But the new studies do prove that the subject is important and advancing in significant ways.

Again, the interesting questions all relate to the matter of causation. Calder is quite right to stress that what have been shown are correlations, which cannot, in the long run, be satisfying. It is like the problem of smoking and cancer; what the correlation means is the paramount issue. We must find ways of understanding what are the causes of allometric relations, and what are the causes of specific examples of deviation from those relations. This is the real strategy for the future and these two books are admirable stepping stones along the way. □

J. T. Bonner is a Professor of Biology at Princeton University.

Science digested

Bryan Silcock

Sci-Tech Report.

Edited by Jon Turney.

Pluto Press/Pantheon: 1984. Pp.386.

Pbk £8.95, \$11.95.

IF THE social responsibility in science movement needs a handbook, here it is: a collection of 170 shortish articles intended to "assess the state of science and technology and locate sci-tech inventions within a wider social and political context". The general approach might be described as left-radical-ecological. Thus nearly all of the contacts and organizations listed at the end of the book are pressure groups, most of them against something or other.

Sci-Tech Report is not a book you can sit down and read. It is far too fragmented for that. But it contains an unusual selection of information that as far as I know cannot be found elsewhere between one set of covers.

The first third, dealing with new technologies of political (in the widest sense of the word) significance — nuclear power, cable television, computers, genetic

engineering, bacteriological warfare — is fairly predictable. But we then move on to such things as accounts of science policy in the main industrialized countries and a few in the Third World as well; articles on aspects of the sociology of science such as the publishing of scientific papers, the ownership of intellectual property and fraud in science; theories of what drives the innovative process in different industries; and discussions of controversial issues, sociobiology and creationism for example, which help to form the public image of science.

The contributions range from the mainly factual to the definitely political. Their style is mostly straightforward and simple, their quality uneven. But anyone with a critical interest in science as an activity and who sees it as more than a dispassionate search for knowledge should find them well worth dipping into.

Sci-Tech Report originally appeared in France. For the English edition some material has been dropped and about 40 new articles added. This was deliberate. I assume that the dropping, in my copy at any rate, of pp. 291–306, and the duplication of pp. 307–322, was not. □

Bryan Silcock is science correspondent of the Sunday Times.

Russian tidings

K.F. Bowden

Ocean Tides: Mathematical Models and Numerical Experiments.

By G.I. Marchuk and B.A. Kagan.

Pergamon: 1984. Pp.292. £36, \$65.

IN THE past 25 years there has been a remarkable revival of interest in ocean tides. This has been due largely to the greatly improved methods of calculation, using modern computers, but also to advances in measurement by deep-sea tide gauges and the developing use of satellite altimetry. Marchuk and Kagan are leading workers in the USSR on the theory and numerical modelling of tides. Their book is in part a monograph describing their own work and that of their colleagues, and in part a general account of the present state of knowledge. While this combination leads to some unevenness in the treatment, both aspects have something of value to offer.

The first chapter gives a clear account of the astronomical and other forces affecting the tides, the equations of tidal dynamics and general features of the methods used to solve them. The next two chapters are more mathematical and are based on the work of the authors and their colleagues on analytical and numerical procedures. While these accounts are likely to appeal mainly to practitioners in this specific field, the fourth chapter is of wider interest and comprises a survey of our present knowledge of the distribution of tides in the world ocean. Following a review of empirical charts, it summarizes the computed co-tidal and co-range charts produced in various countries and discusses a Russian version in some detail. It is interesting to note that, in their preface to the book, the authors express the view that the appearance of more advanced computers will not result in better agreement between computational and observational data. They point to the need to pay further attention to certain physical aspects of the problem.

The last two chapters deal with topics related to ocean tides but which are more specialized and again reflect Marchuk and Kagan's own research interests. In that of the benthic boundary layer, they give a comprehensive review of the subject and describe in particular a theoretical model of their own. The other is on the vertical structure of internal tidal waves which are generated mainly, in the authors' view, by the flow of a stratified ocean over an irregular sea bottom. It is estimated that, in the deep oceans, the rate of transfer of energy to internal tides is of the order of 100 times greater than the rate of dissipation by bottom friction and turbulence. □

K.F. Bowden is Emeritus Professor of Oceanography at the University of Liverpool.

Community questions

C.R. Townsend

Ecological Communities: Conceptual Issues and the Evidence.

Edited by Donald R. Strong Jr, Daniel Simberloff, Lawrence G. Abele and Anne B. Thistle.

Princeton University Press: 1984. Pp. 613. Hbk \$60, £50; pbk \$22.50, £18.80.

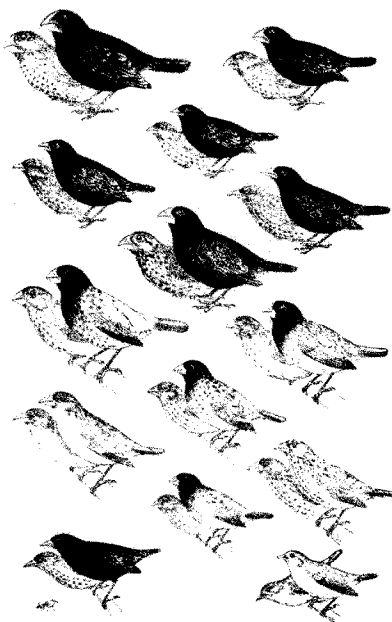
ARE there rules of community assembly and generalizations to be drawn about the factors determining community composition? Or does the group of species populations that occur together at a given location result simply from stochastic processes of colonization and extinction — a random throwing together of species from the available pool? What, moreover, are the relative roles of abiotic influences and of species interactions in determining community organization? These are the central questions of community ecology.

During a period stretching from the 1940s to the 1970s, the conventional wisdom was that interspecific competition for resources dominates the evolution of co-existing species and the organization of communities. The chief proponents of this view — Lack, Hutchinson and MacArthur — considered that species interactions would often proceed according to simple Malthusian expectations, with populations growing until limited by resources and species co-existing only if they exhibit some minimal differences in their resource requirements. The view that emerges in the present volume is that the Malthusian imperative (competitive exclusion of the less efficient of species sharing resources) may often be thwarted because predation, disturbance or abiotic conditions prevent the realization of a competitive equilibrium. If competition is influential, communities are likely to be less diverse in species and, more important, the species can be expected to be more regularly strung out along the resource dimensions of the niche (because of limits to similarity) than their randomly assembled counterparts.

David Lack's work on Darwin's finches, published in 1947, can be used to illustrate several of the threads of the current debate. Lack's aim was to provide an explanation for the evolutionary diversification of *Geospiza* finches in the Galapagos archipelago from a presumed single ancestral species. Believing that the islands were similar in their habitats, he was forced to invoke interspecific competition to account for patterns in morphological characteristics (bill sizes of co-existing species overlap hardly at all) and in species distribution (some species with similar bill sizes do not co-exist). Lack interpreted such regularities as the result of competitive exclusion of similar species or evolutionary shifts generated by competition.

Lack's arguments were persuasive. But while he explained his observations and brought them together into a coherent whole, he did not test his ideas. As Grant and Schluter point out in the present book, until recently most investigators used the concept of interspecific competition both as guidance in the search for patterns and as explanation for them. What they failed to do — and here they are strongly criticized by Simberloff, Connor and Strong, among others — was to test their evidence against alternative hypotheses.

That, however, is much easier said than done. What is needed is a set of hypothetical communities, assembled in realistic ways but in which any effects of



Fitting the bill — Darwin's finches, male and female, about 2/3 life-size. The illustration is taken from the facsimile edition of *Darwin's Finches* by David Lack, published last year by Cambridge University Press in their *Science Classics* series.

competition are specifically excluded. The real community can then be compared against the "neutral models" (so-called) to determine, for example, whether the naturally co-existing species are spaced more regularly along a food resource dimension of the niche than are their randomly assembled counterparts. In the case of Darwin's finches, the neutral model approach of Grant and Schluter leads to the conclusion that the competition hypothesis is, in fact, the best available. However, claims made by other workers that real communities have been structured by competition have often been called seriously into question because the actual species assemblage does not differ significantly from a hypothetical group assembled at random from the available species pool.

The construction of reasonable neutral models is not a straightforward procedure and this is cleverly demonstrated here by Colwell and Winkler. Using a computer

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program called GOD, they first generate a biota, the phylogenetic lineages of which obey specified rules. Subsets of the "mainland biota" then colonize archipelagoes according to a program called WALLACE, and competition may or may not be introduced as a structuring influence. Unlike field workers, Colwell and Winkler *know* whether or not their communities are structured by competitive effects. The interesting and somewhat discomfiting conclusion is that many of the neutral model approaches used in studies of real communities often fail to distinguish any effect of competition even though competition had been included in their program. There are several reasons for this difficulty in detecting "the ghost of competition past", the most important of them being that those species most vulnerable to competitive exclusion are already likely to have been lost from the biota of the entire archipelago. The search for more appropriate neutral models is a major task for the future.

Several of the authors in *Ecological Communities*, notably Brown and Bowers, emphasize that the identification of an apparent pattern and its subsequent comparison with appropriate neutral models should only be the first step. As a result of such analysis, a causal mechanism should be suggested and subjected to experimental testing. Certainly, experiments with real communities are difficult, but they have often helped to determine whether competition (discussed in detail by Brown and Bowers, among others), predation (Dayton), facilitation (Seifert) or stochastic processes (Sale) may

be influencing community structure.

Another message that emerges clearly from the book is that some communities are far from being saturated with species — this alone will limit the general importance of competitive interactions. Both Lawton, in his important work on the insect communities on bracken, and Price, who considers fish parasites, demonstrate the occurrence of "empty niches" in communities.

Robert May, in the opening chapter, questions whether the various themes and approaches are leading us towards a resolution of the factors determining community structure. There is still a long way to go, but *Ecological Communities* must be considered to be a significant landmark on that road. A consensus is emerging (despite the rather vituperative exchanges, recorded here, between Gilpin and Diamond on the one hand and Connor and Simberloff on the other) and the book signals the general acceptance of the need to look critically at the nature of the evidence at our disposal and to give equal weight to the testing of all conceivable hypotheses, not just the one we happen to favour. At present, no one should claim too much. But we can look forward to a gradual emergence of understanding of the types of communities and environments in which particular species interactions are influential, and those in which such interactions are weak, intermittent or non-existent but where, instead, disturbance and stochastic processes predominate. □

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Counsel for chemists

Robert M. Joyce

Guide to the Chemical Industry: Technology, R & D, Marketing, and Employment.

By William S. Emerson.

Wiley: 1984. Pp.330. £35, \$40.25.

Problem Solving in the Chemical Industry.

By R.J. Casey and M.J. Frazer.

Pitman: 1984. Pp.122. Pbk £8.95.

How does a chemistry student assess career opportunities in industry? Few members of university faculties have had enough industrial experience to be helpful, and friends who have recently gone to industry will have seen only a small part of the picture.

In his career counselling guide, W.S. Emerson describes what the chemical industry does and how, the roles of chemists and chemical engineers in industrial research and development, and the career opportunities that may occur. Although it addresses primarily only the development, manufacture and marketing

of organic chemicals, the general principles concerned are applicable to any part of the chemical industry. And while the reader should be able to understand organic structures in order to follow much of the discussion, the style is easy to read and the few errors are not significant.

The author begins with a tour through the principal types of products manufactured in the organic chemical industry, with descriptions of their syntheses, and then moves sequentially through the research discovery, the identification of a potential new product or process, its development and commercialization. The types of industrial research — offensive and defensive — are described, emphasizing that research produces scientific knowledge, not products or processes.

Students should learn that the development of a laboratory discovery is not just a matter of turning it over to some engineers who will simply scale up the procedure. In the 1930s, E.K. Bolton, director of Du Pont's Central Research Department, decided that the nylon his organization would develop must be 66, which could be based on plentiful benzene, rather than 510, which needed castor oil as

a raw material. In doing so, he ignored the opinion of his technical experts that 66 was too intractable, and told them to find out how to make and spin it. They did — fortunately for Du Pont, because IG Farbenindustrie was close behind with 6 nylon, which could be made from benzene. The *Guide* provides case-histories of several developments that were similarly influenced by the technical and economic decisions that must constantly be made as a project proceeds.

Chapters on patents and on unit operations introduce students to subjects that are rarely covered in chemistry curricula. A section on management and marketing gives them glimpses of typical company organizations and of the activities of managers and marketing people, the latter very briefly. This section includes a chapter on reports, which is simply descriptive; one wishes that here the author had taken the opportunity to drive home to students the critical importance of good writing.

The description of career opportunities runs the gamut, with one to two paragraphs on each. And finally, how to get a job. As Emerson points out, selling oneself is the secret to getting a job and to advancing in the organization. In all the author has done well in meeting his aims, and his *Guide* will be worth the time of any college chemistry student who wants to learn something about the chemical industry.

The same cannot be said of *Problem Solving in the Chemical Industry*. After an extended review of the philosophy of problem solving (which includes no chemistry), eight case-histories of chemical problems are presented. The emphasis is on technique: definition of the problem, exploration of various approaches, discarding unpromising ones and arriving at a solution.

However, some of the solutions only define further problems. The case-history on electrowinning of uranium covers in considerable detail seven years of the development of a pilot plant process that was then shelved for economic reasons, leaving one to wonder whether the problem to be solved had been adequately defined. The solution to the problem of disposing of dichromate-sulphuric acid cleaning solution is to me self-evident without considering the alternatives discussed. There are no exercises or problems for the student, and altogether it is rather hard to see who would benefit from reading this book. □

Robert M. Joyce, now retired, was formerly a Research Director for E.I. du Pont de Nemours, Wilmington, Delaware.

● The two volumes in the Scientific American Library reviewed in *Nature* on 15 November (*Constructing the Universe* and *The Second Law*) are in Britain generally available through bookshops. Publisher is W. H. Freeman, prices are £15.95 and £16.50 respectively.

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Application of monoclonal antibodies

This week's product review concentrates on monoclonal antibodies, their production and their applications. New products include a novel gel entrapment technique as an alternative to conventional mass culture.

● Magnetic Microspheres from Molecular Biosystems, Inc. are 1–6 μm diameter spheres of denatured albumin and magnetite coated with protein A. After incubation with an antibody the spheres are used to selectively remove cells or other antigenic species from solution. The Microspheres can be used for either positive or negative selection.

Circle No. 100 on Reader Service Card.

● *Current References Using the ABC Immunoperoxidase Procedure-2*, is the second in a series of reference lists compiled by Vector Laboratories. The 120 recent publications cited in this latest list include staining of paraffin and frozen sections, cytocentrifuge preparations, solid-phase enzyme immunoassays, nitrocellulose blots, and pre- and post-embedding electron microscopy using monoclonal antibodies and lectins. A key covering a range of specific applications is also included.

Circle No. 101 on Reader Service Card.

● Genzyme Corporation of Boston, Massachusetts has completed a licence agreement with Dartmouth College (signed on 27 September) to produce and sell monoclonal antibodies to interleukin-2 from a cell line developed by Dartmouth. The monoclonal anti-interleukin-2 joins Genzyme's already extensive line of research biologicals which includes interleukin-1, interleukin-2, recombinant interleukin-2, interleukin-3, GM-colony stimulating factor and collagen 2.

Circle No. 102 on Reader Service Card.

● A panel of five new monoclonal antibodies against hepatitis B surface antigen is now available from Sera-Lab of Crawley Down, Sussex. These monoclonal antibodies, MAS 103, MAS 104, MAS 105, MAS 106 and MAS 107, have proved useful in a sandwich solid-phase radioimmunoassay for HBsAg. In particular, the use of MAS 105 as both capture and tracer antibody results in a very sensitive assay system.

Circle No. 103 on Reader Service Card.

● A new brochure, entitled *Immunochemicals and Related Products*, will be available from Boehringer Mannheim Biochemicals in January 1985. Product areas to be included in the brochure are: monoclonal antibodies; protein A products; affinity isolated antibodies; reagents for enzyme immunoassay; immunohistochemistry reagents; and cell culture products. The brochure also includes details on various immunology techniques.

Circle No. 104 on Reader Service Card.

● The Swiss company Robapharm AG of Basle has launched a range of rare monoclonal antibodies against human tissue antigens with special emphasis on cells of the reticuloendothelial system. Currently available are antibodies which distinguish vascular endothelial cells of different organs (such as lung versus liver), subtypes of tissue macrophages (resident versus inflammatory) and which are specific for smooth muscle cells. The antibodies represent new tools for research on the vascular endothelium and on mononuclear phagocytes and may be useful in the diagnosis of endothelial cell tumours, pathological changes of the vessel wall (atherosclerosis) and various cell infiltrates.

Circle No. 105 on Reader Service Card.

● The Immunex Corporation of Seattle has entered into a licence agreement with Becton Dickinson & Company for the sale of research and diagnostic products based on a monoclonal antibody developed by Immunex scientists. Becton Dickinson introduced the first products based on the antibody in late September. The agreement covers an antibody that quantifies the number of activated T-cells — antibody to interleukin-2 receptor. Three products are available based on the antibody, a purified form and two fluorescent conjugates.

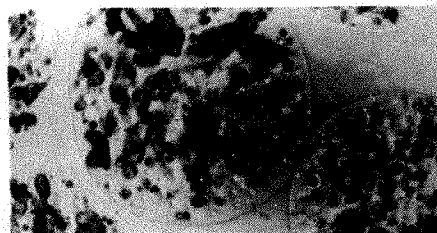
Circle No. 106 on Reader Service Card.

● The UK company, Tissue Culture Services Limited has signed an agreement with Ventrex Laboratories, Inc. of Portland, Maine, giving TCS manufacturing and distribution rights to HL-1, a serum-free cell culture media product, in either liquid or powder form, for the culture of hybridomas and certain other cell types. Use of HL-1 with a completely defined composition and extremely low protein content (less than $50 \mu\text{g } \mu\text{ml}^{-1}$) facilitates the *in vitro* scale-up of monoclonal antibody production, and simplifies or eliminates purification steps.

Circle No. 107 on Reader Service Card.

● Myxothiazol, a potent inhibitor of the mitochondrial respiratory chain, is now available from Boehringer Mannheim Biochemicals. This highly toxic antibiotic is isolated from the gliding bacterium, *Myxococcus fulvus*. Myxothiazol is similar to antimycin A, in that both inhibitors bind to cytochrome *b*. However, current evidence suggests that myxothiazol binds to the (*b*_L) haem centre, while antimycin A binds to the other form (*b*_K). Acting together, these inhibitors will completely block electron transfer to cytochrome *b*.

Circle No. 108 on Reader Service Card.



The 1-mm diameter beads of the Geltrap system for monoclonal production.

● The new Geltrap system from Karyon Technology, Inc. makes possible the large-scale production of monoclonal antibodies and other mammalian and plant cell products. The gel entrapment process offers a simple, flexible and cost-effective alternative to mouse or conventional mass culture techniques. Other applications of the Geltrap system include production of hormones and alkaloids, enzyme and bacterial immobilization, and sustained release therapy.

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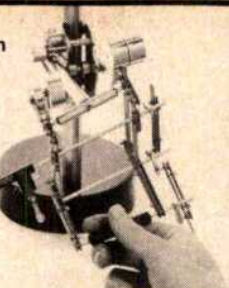
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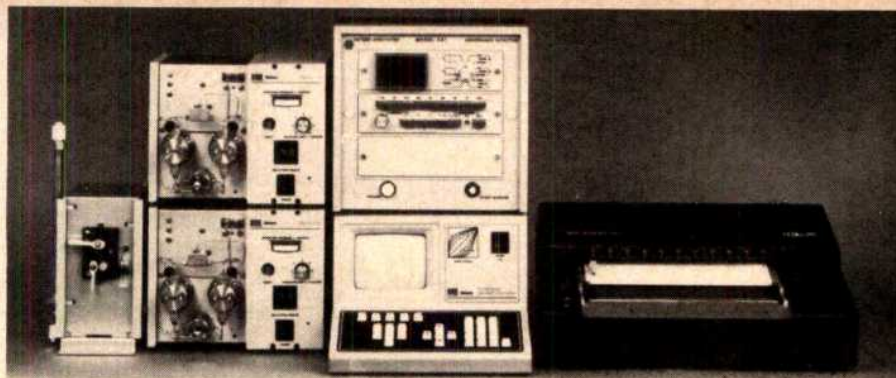
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Monoclonal antibodies can be isolated within 30 minutes on this HPLC system from Waters.

● An HPLC system designed to isolate monoclonal antibodies from ascitic fluids and cell culture supernatants has been introduced by the Waters Chromatography Division of Millipore Corporation. The HPLC system uses high-performance anion exchange chromatography to separate the monoclonal antibody from the primary constituents of ascites and cell culture supernatants — albumin, transferrin and host immunoglobulins. After sample preparation using microfiltration (for ascites) or ultrafiltration (for tissue culture), the separation is typically performed in less than 30 minutes. To monitor the performance of the isolation procedure, the same HPLC system can be used to assay the homogeneity of fractions by gel filtration. The system can be easily upgraded for automated sample injection.

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● Miles Scientific now offers four monoclonal antibodies to human IgG heavy-chain determinants. These undiluted antisera offer maximum flexibility for those laboratories that want to further purify the antibody for conjugation to enzyme, fluorescence or radioisotope labels for immobilization on a solid-phase support. The products are characterized in sensitive EIA assays and show broad reactivity to a large group of purified normal and myeloma IgGs of all four subclasses.

Circle No. 111 on Reader Service Card.

● Sanbio b.v. of the Netherlands, working in cooperation with the University of Leiden and other Dutch institutes, has produced a large range of monoclonal antibodies for indirect immunoperoxidase staining in tissues and ELISA techniques. The range includes anti-human keratin, neurofilament, hCG, FSH, and glia fibrillary acidic protein. Anti-human desmin, vimentin, thyroglobulin and blood vessel endothelium are also available.

Circle No. 112 on Reader Service Card.

● A newly available monoclonal antibody radioimmunoassay kit for human gamma interferon provides a convenient and reliable assay method. The first product to be launched by IMRX Corporation, a joint venture of Centocor, Inc. and FMC Corporation, the IMRX Interferon-Gamma RIA is a two-step "sandwich" solid-phase radioimmunoassay.

Circle No. 113 on Reader Service Card.

● Serotec Ltd is a specialist supplier of monoclonal antibodies and polyclonal antisera and has just introduced a series of 150 new products. The new range includes: sheep antisera to human, rat and mouse immunoglobulins conjugated to glucose oxidase which reacts to give a deep blue stain in immunohistology and in ELISA, and beta-galactosidase, including second antibodies; monoclonal antibodies against human proteins, such as T-helper lymphocytes, T-suppressor lymphocytes, sub/cytotoxic T cells and B lymphocytes; and monoclonal antibodies against rat proteins such as MHC I antigens and MHC II antigens (1a).

Circle No. 114 on Reader Service Card.

● The Nottingham-based company Hybritech Europe offers a range of isotopic and non-isotopic kits for the measurement of human beta chorionic gonadotropin (hCG) in urine or serum. Tandem-R hCG utilizes two distinct monoclonal antibodies in a solid-phase immunoradiometric assay. Tandem-E hCG is a non-isotopic solid-phase immunoenzymatic assay for hCG using two distinct monoclonal antibodies.

Circle No. 115 on Reader Service Card.

● Monoclonal antibodies against 4-azido-2-nitrophenyl (NAP) hapten have been introduced by Sera-Lab. These antibodies, MAS 089 and MA 090, are of value in the study of immunoregulation. NAP hapten is a photolabile label and can be attached covalently to the antigen binding sites of these antibodies.

Circle No. 116 on Reader Service Card.

● Becton Dickinson Immunocytometry Systems has announced what is claimed to be the first comprehensive research panel for lymphoma subtyping. The panel consists of six well characterized monoclonal antibodies plus accessory reagents for immunoperoxidase staining of frozen tissue sections. The monoclonal reagents include: anti-leukocyte (a pan-leukocyte marker), multi-clone anti-T cell, anti-LEU-14 (B cells), anti-IgM, anti-kappa and anti-lambda.

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Students on the poverty line

The British government will this week be forced to back down from last month's proposals for modifying students' grants. It should take the chance to reform the system.

SIR Keith Joseph's attempt last month to provide a modicum of help for the British academic research enterprise seems to have blown up in his face. The plan (see *Nature* 15 November, p.185) was to add £14 million to the income of the British research councils, and to give the University Grants Committee an extra £10 million to spend on equipment, financing the operation within the government's ceiling on public spending by reducing the value of the maintenance grants paid to university students. What Sir Keith seems to have overlooked, and what his Cabinet colleagues failed to spot when they approved his part of last month's package of public spending plans, is that the uniquely British system of university maintenance grants has become part of the British way of life, and part of the middle-class way of life in particular. By last weekend, the government was in serious trouble. Nearly half of its back-bench supporters in the House of Commons had declared themselves against the new arrangements, much to the satisfaction of the government's formal opponents. Most British newspapers have denounced the changes planned in language which is a rich blend of invective and humbug. But even the president of the Royal Society, speaking at the society's annual dinner last Friday with Sir Keith Joseph sitting as his guest a few feet away, offered the opinion that while the extra £24 million for research would be welcome, he regretted that it had to be won at the expense of student grants. Students are no doubt surprised to find how many friends they have.

Joseph, the Secretary of State for Education and Science, will be less surprised by these demonstrations that he has so few friends. He has had a long run of unpopularity (first at what was then the Department of Industry) going back to 1979, at least in part on the dubious ground that he inclines to abstract argument, being a kind of theoretician among politicians. It would nevertheless be a shame if, during this hullabaloo about student grants, it is forgotten that, in two consecutive British budget years, he has done his best to win more resources for the support of research, last year by the simple device of asking for more (and being refused), this year by shifting funds from one line to another within his own department's budget. Latterly, the minister has also been shaping up to tackle the obvious problems of British secondary education (specialization and the persistence of sloppiness) in ways from which his predecessors have mostly shrunk. Against the odds of a few years ago, it would be a misfortune if the government seeks to solve its immediate difficulty (how to back down without seeming to do so) by executing the traditional penalty for the bearers of bad tidings, for example by sending Sir Keith Joseph off to the House of Lords.

Restrictions

What the fuss this week should be about is not, in any case, the particular amendment of the students' maintenance scheme put forward by the minister but the scheme itself, which is a monument to the optimistic 1960s, when the expansion of British higher education was set in train. The principle is that young people should not be deterred from seeking higher education by the cost of doing so, for which reason local authorities are required by law to pay the tuition fees of British students at universities, polytechnics and other higher education establishments, and also to provide them with a grant to cover the cost of maintenance which, this year, ranges from £205 a year to nearly

ten times as much, depending on parental income. One of the consequences of this arrangement (which allows local authorities to reclaim the cost of grants from central government) was the imposition in 1981 of iniquitous and uneconomic quotas restricting student numbers at universities. The changes now proposed are adjustments to the way in which student grants are calculated from parental income which will have the effect of giving less to students with parents whose income exceeds £13,000 a year (after certain deductions). The new proposals do, however, break new ground in that they will require the offspring of well-off parents (with combined incomes above £20,000 a year) to contribute towards tuition fees (up to a maximum of £520 a year). The new scales will apply to all students in higher education at the beginning of the next academic year, those already embarked on academic careers included.

Hardship

This ramshackle system will be made only marginally more inequitable by the changes now proposed. So long as "mandatory" grants remain, British governments will feel compelled to hold opinions about the numbers of students higher education establishments should be teaching (which, strictly speaking, is none of their business). At the same time, the numbers of students suffering financial hardship will be increased as greater numbers of parents decline to make the financial contribution that the system implies is needed. And the proportions of young people from poor homes seeking higher education of any kind is likely further to decline, which is ridiculous at a time when everybody (the government included) agrees that too small a proportion of young people in Britain are acquiring a modern skill. The remedy for all these evils will be found not by tinkering with the present system but by redesigning it. This is how the government should set about that task.

First, to put out the immediate fires, the distasteful retrospective element in the new proposals should be dropped; students part way through a course should be allowed to complete it under the rules understood when they began. But the government should also recognize that the sums of money it is assuming middle-class families in impoverished Britain will find to put their sons and daughters through higher education will for many be substantial fractions of an annual income. Unfortunately, after a long flirtation with the idea, the government has now set its face against proposals for loan schemes to help finance higher education; it should now reconsider that decision. It should go further, and acknowledge that one of the reasons for this week's middle-class revolt is that many people have suddenly woken up to what they should have known all along, that contrary to the appearance suggested by the mandatory grants scheme, British higher education is not (and has not been for a long time) free. What needs now to be done is to hammer out an equitable basis for meeting the costs.

The first need is for a decision on tuition fees. Should British students pay, or be excused? The way in which this question is answered is vital for the health of the university system as well as for the welfare of students, for fee income is potentially one of the ways in which British universities can hope not to be entirely dependent financially on the government. But what passes for a "tuition fee" at present is an entirely notional sum of money

which is, to what should be everybody's surprise, fixed not by universities but by the government (which repeatedly adjusts it up when it wishes to augment universities' sense of freedom and down, as in 1981, when it wishes to cramp them in). What would now best suit the universities is an understanding that they should be free to charge what tuition fees they choose, and that they should keep what they collect. Ideally, the fees asked of students should be related to the marginal cost of education (and not to the average, which must be substantially greater), but if the years ahead may see some universities hard pressed to find students, they may need the freedom to charge what the market will bear.

The question of whether governments should, in principle, pay the cost of maintaining students in higher education is more complicated, and goes back to the report of the Robbins committee which, in 1963, launched the expansion of the British university system with the doctrine that access should be open to all school-leavers who are qualified to take advantage of it. Especially because so many of the new British universities are sited miles away from their catchment areas, the assumption has grown up that students will ordinarily travel to some distant place if they enter higher education, and successive governments have found themselves embroiled in tedious arguments with the National Union of Students over the proper level of the "travel grant" by which the ordinary maintenance grant is supplemented. One consequence, from which the British university system as a whole has suffered, is the erosion of the regional connections of universities such as the red-brick creations of the nineteenth century. It would not be a serious setback for the British system if the principle were now followed that student maintenance should not be a public charge. To protect universities that are vulnerable because of their geographical isolation, there would no doubt have to be a transitional arrangement of some kind.

Outside Britain, it is bound to seem strange that the provision of grants to students is a serious issue. Almost everywhere, students are constrained in their choice of higher education establishments by the costs not merely of tuition but of maintaining themselves independently. Can Britain, with its perpetual money troubles, afford to be different?

One practical consequence of such a move towards economic realism would be serious hardship among students, many of whom already live in too much squalor, perhaps waiting for parental contributions that do not materialize. Moreover, British students are less able than those elsewhere to earn money to help with their costs, partly because of the economic climate but also because of trade union restrictions, while most universities have no significant endowment funds with which to help able people in trouble. That is why British policy on students can be transformed to what it should be only by equipping universities financially to be able to do their duty to their students.

The problem of students from poor homes is different, serious but soluble. Entry to British higher education is still skewed against the children of the less well off, with the consequence that too little is made of the national pool of talent. The needs of these potential students are theoretically catered for by the present system of maintenance grants but, as the figures show, the outcome is inadequate. The simple remedy is to replace the present system by one of scholarships awarded to able school-leavers and others which, at their most generous, would cover not merely the bare cost of maintenance but some part of the opportunity cost of higher education (the wages forgone), but which would be tenable anywhere, not simply at the institution that happens to offer a student a place. Beyond the resolution of that problem there are other conundrums to be tackled. Slowly, in the past few years, British governments and universities have awakened to the need and opportunity for using existing educational plant for training persons of mature years, many of them already in jobs. But there are at present only disincentives (tuition fees out of taxed income, for example) to its efficient use. That is also a problem that needs solving. If the trouble Sir Keith Joseph has brought down on his government's head should have the effect of spurring a search for better ways of enlarging participation in higher education, it may yet be worthwhile. □

Japanese uncreativity

Japan's new strategy for research is more a recipe for continued success than for creativity.

JAPAN'S obsession with creativity is a curious phenomenon. Merely moderately successful nations are usually distinguished by their complacency. Japan, by contrast, after three decades of spectacular economic success, seems perpetually down in the dumps, with its conviction that Japanese technology is in reality derivative, and that its gigantic technical effort lacks creative flair. Is it possible that Japan's competitors in the West, beaten onto the ropes though they may be the technical achievements of Japanese industry, have been able to ensure that Japan does not fully enjoy its success by suggesting that there is something missing?

The point has been endlessly discussed in Japan in the past five years, but will not be settled with the publication this week (see facing page) of the blueprint for future research drawn up by the Council for Science and Technology in Tokyo. There is a grain of truth in the common Japanese assumption that many of the products with which Japan has swept the world are derived from machines already built elsewhere. But so what? From ships to video-recorders, Japanese technologists have been able to design and manufacture high-quality products more cheaply than others, and at the same time make sure that they function reliably. If the ingenuity is not creative, many technologists elsewhere will be wondering what is meant by creativity.

The more substantial sense in which Japan is technically uncreative is in the contribution of the university system to basic science, but the reasons are now familiar. First, the rigidity of the academic institutions, and the parsimony with which they are supported, drives energetic academics into applied science and into partnerships with industry. Second, students march predominantly into the academic engineering departments. Righting this imbalance would require long-due changes in the academic structure, which the science and technology council has plainly considered outside its terms of reference. Indeed, the list of objectives accompanying this week's strategic plan is not so much a recipe for creativity as a shopping list for tomorrow's high technology that could be found in most other places in the West. To be sure, Japan may succeed where others fail, but that is a very different matter. □

Damming the water

Are the British and French governments losing an opportunity?

WHAT follows is not a joke. For at least two hundred years, but at different times, the English and the French have been suggesting proposals for a permanent link across the 21 miles of water that separate them. Most schemes have turned on the construction of a tunnel. In the 1960s, bridges briefly became fashionable. Now, the favoured way of spanning the Channel is to build a composite structure, part bridge and part tunnel. To judge from the Anglo-French meeting in Paris last week, the remaining obstacles to a permanent link are merely legal and financial.

Nobody, however, has seriously considered what must be the most obvious way of spanning this narrow and shallow stretch of water, the building of a permanent dam across the Channel. The advantages are obvious (see *Nature* 219, 991; 1968). It would be possible to generate endless electricity, the Dutch would no longer have to worry about their dykes and it would be possible to walk across, as at the glacial maximum 18,000 years ago. The disadvantages are few, the most obvious of them being the disturbance caused to shipping, most of which could just as well go round the north of Scotland. The cost of such a project has never been determined, but could well turn out to be surprisingly small. Although megaprojects are now out of fashion, should not the British and French governments look seriously at this ideal before committing themselves to a clumsier alternative? □

Japanese research

Strategy for next decade aims at more creativity

Tokyo

THE Japanese government last week unveiled a major new report mapping out its fundamental policies for the promotion of science and technology over the next ten years. Stress is now being laid on the encouragement of creativity in science — apparently in response to Western criticism — and, along with it, greater international exchange and cooperation and the development of science in “harmony with human society”.

The report, entitled *Tackling changing conditions; Comprehensive and fundamental policies for long-term promotion of science and technology*, is the work of the Council for Science and Technology, the highest policy-making body in the nation, whose chairman is the Prime Minister. Some 150 experts drawn from the universities, government research laboratories and private industry helped to draw it up, together with the heads of the science-based ministries and the Science Council of Japan, the academic representative body, who have permanent seats on the council.

In its 24-year history, the council has produced only 11 reports. Each has tried to set a policy framework and to define key research areas so that the ministries concerned with science can implement detailed policy in accord with the government's overall aim.

To carry out the policies suggested by the new report, increased expenditure will be required — and increased expenditure certainly seems to be what Japan has been getting. The report reveals that Japan's research and development expenditure, most of it provided by private industry, will reach 3 per cent of national income this fiscal year (over 2.8 per cent of gross national product) after four years of growth at a remarkable 0.2 per cent of national income per year. Only West Germany among the industrialized nations can claim to be spending a greater percentage of its national income on research, but the lead should pass to Japan within 2–3 years.

In calling for increased creativity, the report accepts the view that “up to now Japan has taken in foreign science and technology, improved and developed it in a highly efficient response to social and economic needs to establish its present developed (industrial) state...”. But now the call is for measures to smooth the way for Japan to become one of the top nations in basic research. Several trends are seen as making this urgent: the so-called “soft revolution” — the accelerating pace of the information sciences, of which computer software development is one part, where Japan has not yet made a name for itself;

the ever closer links between basic and applied research required to make possible further “miniaturization” in electronics and sophisticated control of structure and function in materials; and the increasing influence science and technology are having on human life styles.

To increase creativity, better links are called for between university and industry — noticeably absent so far in Japan. No concrete measures are suggested, however, and it is difficult to see how the present trends towards increasing reliance on private sector research will be changed. Calls are made for more efforts to train researchers in biotechnology and advanced electronics in view of an expected shortfall that could limit basic research activities.

“Internationalization” is also called for — the first time a council report has clearly done so. Foreign participation in government research projects and foreign use of government facilities are possibilities mentioned. What the report does not mention, however, is that the current trend is towards increasing competition in basic research programmes (such as the fifth generation computer project) and attempts to launch cooperative projects at the various summits have not met with success: some revolutionary steps will be needed to bring about international cooperation on any significant scale.

In contrast with the generalities of much

of the report, which will require interpretation by the ministries concerned, key research themes where Japan must now make major basic research efforts are more clearly mapped out. These include research on new materials, electronics and information sciences, life sciences and earth sciences (see below). Many of the areas of materials sciences mentioned have already become the focus of long-term projects sponsored by the Science and Technology Agency and the Ministry of International Trade and Industry (see *Nature* 305, 373; 1983). Added here are calls for increased basic work on reaction theory and mechanisms to make possible the prediction of materials with new properties — welcome news to many university research groups.

In electronics and information sciences, areas likely to be commercialized in the near future are avoided. Stress is laid on fundamental projects such as production of the components necessary for optical circuits and eventually an optical computer, and steps towards a new science of “bioelectronics”. Several electronics companies have already moved into this futuristic field, setting up groups to research, in particular, Langmuir–Blodgett films.

Life science themes to be promoted include, unsurprisingly, all the fundamental technologies that will some day have applications in biotechnology. In the earth sciences, remote sensing and the building of new satellites and data networks lie at the heart of themes aimed to make better predictions of the natural disasters — earthquakes, typhoons and floods — to which Japan is prone, and to enable natural resources, including sources of geothermal heat, to be detected more easily.

Alun Anderson

Japan's strategic objectives

AMONG the basic research themes defined the report are:

Materials science

Materials produced under extreme conditions such as ultrahigh pressure, temperature and magnetic fields-strength; artificial lattice and amorphous structures; surface properties and new ways of analysing them; interaction of materials with X-ray, heavy and light ion beams; development of free-electron, excimer and chemical lasers; materials with mixed atomic and molecular bonding with new properties (organic superconductors); materials design by studies of unstable reaction intermediates and bonding properties, including development of associated computer stimulation and database facilities; control of form and purity as in ultra-fine particles, highly ordered structures and films; composite materials and molecular hybrids; materials mimicking natural materials, such as artificial bone; new analysis and evaluation techniques.

Information sciences

New devices, concentrating on Josephson

junctions, image processing and intelligent sensor devices; optical electronics; bio-electronics and biocomputing; knowledge management; fifth-generation computing and its connections with mathematics, symbol manipulation and psychology.

Life sciences

Gene regulation extended to cell regulation; extraction, analysis and synthesis of DNA; design, modification and synthesis of functional proteins; chromosome engineering, including modification and automatic mapping; cell and organelle modification; altering components of cells and tissues and development of the whole organism.

Earth sciences

Atmosphere-sea interaction, unusual weather conditions and typhoon generation, requiring new observation satellites and Earth-based networks; mantle dynamics, earthquakes and volcanic activity; new satellite-borne sensors and laser ranging equipment, new data networks and new Earth resource satellites.

Alun Anderson

Anglo-French cooperation

Technology dominates summit

AFTER the bitter battles over British contributions to the European Community budget and almost every other kind of acrimony between France and Britain, the British Prime Minister, Mrs Margaret Thatcher, and the French President, M. François Mitterrand, last week made up their differences at a meeting in Paris at which agreements on technology and research played an unusually prominent role.

M. Mitterrand was the cooler of the two. He spoke on Friday of "an excellent spirit of cooperation". Mrs Thatcher, more fulsome, spoke of "a completely new atmosphere... it is just a totally different bilateral from any we have had before... more constructive, more cooperative". With the budget issue out of the way, she wanted to suggest, real Franco-British cooperation can begin.

Technology came up as the list of major projects on which Britain and France have either agreed in the past 12 months or hope soon to agree. The highlight was the Channel Tunnel, or "fixed Channel link" as it is called in France to include the possibilities of both tunnel and bridge.

The two governments, no doubt aware that the tunnel was first suggested in 1751, emphasized the "urgency" of the studies now under way that will identify the political structures under which the "link" can go ahead. Both sides have agreed that the link must be paid for by private funds, but potential backers have asked for guarantees that construction will not be halted for political reasons.

The tunnel apart, the Franco-British summit raised the following issues:

- Esprit, the European cooperative research programme on information technology, which is felt greatly to have improved bilateral links between French and British companies.

- Fast reactor development, where outline agreements among European utilities, research organizations and fuel-cycle companies were reached this year following a political umbrella agreement in January. (But details of exactly who does what and where have still to be agreed.)

- The Versailles summit programme, under which "quite fruitful" Franco-British work is going on in biotechnology and food technology.

- A Chinese pressurized water reactor for Guangdong, under development jointly by Framatome of France and General Electric Company of Britain. "We hope, both of us, to get substantial orders for that power station", said Mrs Thatcher last Friday.

- Direct broadcast satellite (DBS) transmission standards, where bilateral discussions are well advanced to avoid the dual standards (PAL and SECAM) that have plagued the European domestic television market.

- The European A320 Airbus, on which Britain is to collaborate more fully than on the earlier A300 and A310 versions, it is claimed in Paris.

- A military helicopter engine (RTM 322) under joint development between Rolls-Royce and Turbomeca, a third-generation antitank weapon (with Germany) and a multi-launch rocket system (also with Germany).

Telecommunications and space projects were also mentioned, although progress in

opening the British telecommunications market to the French (and vice versa) has not gone as fast as the French would have liked, and space agreements must wait for the European space summit in Rome next January.

The meeting also postponed until next year a Franco-British "association" for discussing the exchange of results between universities and industry (see *Nature* 1 November, p.4) which will be debated at a bilateral meeting in February. Meanwhile, soundings are taking place to determine exactly what each side would like to get out of such an institution, and how it would work in practice.

Robert Walgate

Polish science budget

Paper generosity decreed

Warsaw

POLAND'S science budget, at present one of the lowest in Europe in relation to national income, is to be substantially increased. Last week, the academic secretary of the Polish Academy of Sciences, Dr Zdzisław Kaczmarek, told the presidium of the academy that next year the government would increase the academy's "science" budget by 26 per cent, and that financial support for the academy would be further strengthened by an allocation of 1,000 million zloty from the central technical economic fund. This, said Dr Kaczmarek, would allow more money to be allotted to research. Wages within the academy network, moreover, would be increased, on the average, to 10 per cent above the national mean, rising to 30 per cent above the national mean in the next three years.

Dr Kaczmarek's announcement has been received with a mixture of relief and

scepticism. Poland at present spends only 2.5 per cent of the national income on science (including the university and industrial research sectors), and any increase is clearly welcome. Many scientists, however, feel considerable doubt as to whether the wage rises can be as substantial as promised. One researcher said that her own monthly salary, 7,000 zloty, is not only far below the national average of 17,000 zloty, but well below the salary of those doing similar work in the universities.

There is also some scepticism about the new "Committee for Matters of Science and Technical Progress", which is intended to have responsibility for research areas of science of particular importance for the national economy. Many of the older generation of scientists see it as a return to a similar body of the 1960s, when the academy's powers were considerably less than at present.

Vera Rich

No cancer cure

Washington

A BATTLE is in prospect between the Food and Drug Administration (FDA) and the National Cancer Institute (NCI) over whether the government should throw the book at the Kellogg cereal company for its advertising campaign that promotes All-Bran cereal as a cancer preventive.

Kellogg's television and print advertisements running since the beginning of October state that "the National Cancer Institute [NCI] believes a high fiber, low fat diet may reduce your risk of some kinds of cancer", and then proceed to extol the virtues of All-Bran's high-fibre content. The print advertisements also tell readers how they can obtain additional cancer prevention information from NCI.

NCI officials, who have made diet modification a key component of their plans to cut cancer mortality by half by the year 2000, appear to support Kellogg's efforts; they particularly seem to like an offer by Kellogg to turn over to NCI the backs of their cereal boxes for additional

cancer-prevention messages. In a report to Health and Human Services Secretary Margaret Heckler, Assistant Secretary for Health Edward Brandt noted that "this effort by the Kellogg Company will infuse millions of dollars of private sector funds into the promotion of cancer prevention".

But FDA meanwhile has been looking



into the possibility that the Kellogg advertisements violate federal law. While avoiding direct comment on the Kellogg case, Mark Novitch, deputy commissioner of food and drugs, recently told a food industry meeting in Washington that "current FDA rules prohibit any labelling [on foods] that makes health claims".

Stephen Budiansky

Cohen-Boyer patent

Licensees ponder weaknesses

Washington

IN the aftermath of the long-awaited grant of the Cohen-Boyer patent in September, it is becoming increasingly clear that serious questions about the patent's validity remain unresolved. The opening of the complete file has in fact brought to light new questions, and Stanford University may now be reluctant to enforce its patent in the courts for fear of opening these issues to new scrutiny, possibly leading to judicial invalidation of the patent.

The September patent, which covers recombinant DNA molecules used in the cloning process developed by Stanley Cohen and Herbert Boyer, was issued to Stanford University and the University of California after a prolonged negotiation with the Patent Office. Stanford, which had initially waived its right to secrecy during this process, abruptly reversed course in November 1982 after the Patent Office tentatively rejected the claim and began asking uncomfortable questions.

It now turns out that shortly after asking that the file be closed to public scrutiny, Stanford's attorney, Bert Rowland, was told by the Patent Office that the case would be turned over to its fraud examiners; the examiners in spring 1984 concluded that Rowland and the applicants had committed a "clear violation of their duty to disclose to the Office information they are aware of which is material to the examination of the application". Because these violations were found to be the result of something less than bad faith or "gross negligence", however, they did not affect the ultimate issue of the patent.

The violations concern a scientific paper published by Cohen in 1977 that contradicts the earlier patent application description of how to make the key cloning vector pSC101, a plasmid. The Patent Office learned of the article only in June 1982, just as it was preparing to approve the patent; and the source of the information was press accounts of a critical article by Albert Halluin, now a patent attorney for Cetus Corporation. In the autumn of 1982, Rowland admitted to the Patent Office that he had known about the 1977 article when it had appeared, but had "no recollection" of why he failed to bring it to the Patent Office's attention. The fraud examiners' investigation further revealed that Rowland had written to Stanford's patent administrator in 1977 suggesting that the Cohen article might render the patent's description of how to make pSC101 "non-enabling" and asking whether they should revise the application accordingly.

Another apparent inconsistency is that in response to questions from the fraud examiners, Cohen early this year stated to the Patent Office that he had not appreciated the significance of the 1977 article with respect to the patent "until the

significance was explained to me at the time of my preparation of my declaration of April 20, 1983". In August 1982, however, Cohen denied reports in *Nature* (see 298, 595; 1982) and elsewhere that the 1977 paper admitted an error in the patent description. Rowland's declaration in autumn 1982 admitted to the Patent Office that pSC101 "could not be prepared in accordance with the working example" in the patent.

The real question for Stanford now is how many companies will continue to pay the \$10,000-a-year licence fee for the patent, due next February. Sixty-six companies are currently enrolled. The weakness of the patent is clearly weighing on the licensees' minds. An appellate court decision within the past month has con-

siderably toughened the definition of fraud, and if this standard had been applied to the Cohen-Boyer patent, it would probably not have been issued. The new tough standard would be applied by the courts now in any legal challenge to the patent's validity.

It is thought very unlikely that any company will go to the expense of mounting such a challenge. But Stanford itself would be opening the door if it goes to court to enforce the patent in an infringement action. It is thus likely that some companies may choose simply to ignore the patent, in effect daring Stanford to respond.

And even on the face of the patent claims, Stanford's position is not so strong as it would like it to be. The patent is limited to prokaryotes. And the admitted flaws in the pSC101 description remain in the earlier patent covering the cloning process, thereby raising doubts about that patent's validity.

Stephen Budiansky

Dangerous pathogens

Illegal US bug trade halted

Washington

TWO Canadians were arrested by Federal Bureau of Investigation (FBI) agents in Buffalo, New York State, last week as they attempted to collect botulinum and tetanus cultures that they had ordered under an assumed name from the American Type Culture Collection (ATCC). Neither of the two is a scientist, a fact that has fuelled speculations of a possible extortion scheme or terrorist plot.

The incident has also served to highlight the virtual absence of regulations governing the sale and acquisition of highly infectious or toxic agents. Federal regulations restrict only the importation or transfer of foreign microorganisms; indigenous microorganisms can in principle be sold freely to anyone. The two Canadians in fact obtained a tetanus culture from ATCC in September without incident.

It was only after they placed a second order that ATCC officials apparently became suspicious and approached FBI. The bureau found that the name and address used by the men were fictitious, and asked ATCC to process the order, substituting inert samples for live cultures. The Canadians were presumably going to carry the material across the border themselves, avoiding the customs problems of a direct shipment to Canada. When they attempted to pick up their order, FBI agents were waiting for them.

The only crime the two men have been charged with is wire fraud, for having used a false name in placing the order over the telephone. According to the FBI office in Buffalo, the men did not attempt to pass themselves off as scientists, although they did tell ATCC that they wanted the material for unspecified "research". FBI has identified the men as a Bell Canada employee and an Ontario Industrial Develop-

ment Corporation employee. No motive has been established.

ATCC is the major germ-plasm reference bank and supplies culture materials to researchers at or below cost; according to Pierre Daggett, acting director of ATCC, an internal safeguard system screens out people who are not qualified researchers and ensures that they do not receive infectious material. ATCC distributes 60,000 cultures per year. The regulations governing foreign microorganisms, which are administered by the US Department of Agriculture and the Public Health Service, require researchers to obtain a permit.

Both tetani and botulinum owe their lethality to toxins that they produce while growing under anaerobic conditions. Tetanus toxin is not normally harmful when ingested; botulin is a potent toxin when ingested or inhaled.

Toxin experts said last week that a botulinum culture of the type supplied by ATCC would probably be a mixture of spores and live cells, and possibly a small amount of toxin. Anyone with a basic knowledge of microbiological techniques, however, would have little difficulty in growing larger cultures and producing considerable quantities of toxin. On the other hand, any microbiologist would also have little difficulty in isolating botulinum or tetani from the environment.

Dr Michael Gill of Tufts University discounted the possibility that botulin could be used as a terrorist weapon: "The old scenario of putting it into a reservoir is not going to work". The bacteria would not grow in oxygenated water. But speculation is now centring on a possible extortion scheme, in which commercial products would be contaminated, or perhaps merely the claim made that this had been done.

Stephen Budiansky

UK research councils

Cake-cutting done

THE science and medical research councils are the chief beneficiaries of the extra £14 million the British government has made available for the coming financial year. The allocation of the extra funds was to be disclosed in the annual "forward look" of the Advisory Board for the Research Councils (ABRC), whose publication has been brought forward to help the Secretary of State for Education and Science to defend himself against his critics in the House of Commons who are complaining at the way the extra science money has been quarried from the budget for students' maintenance grants.

The ABRC document has something of the character of a ritual dance. To begin with, it records its own estimate of the funds that would be needed to take advantage of new opportunities for research that have been presented in the past year, reckoning that these would cost an extra £35.5 million in 1985-86. On the other hand, ABRC says elsewhere in the same document, the extra £14 million for next year (which is £16 million if account is taken of the way in which the government compensates the research councils for pay settlements) "will allow many important research projects" to continue that would otherwise have been allowed to lapse.

Much of the extra money for the coming financial year will be spent on what is called "restructuring", a euphemism for persuading researchers to retire early when the institutes at which they work are closed. ABRC had asked for £9 million in 1984-85, but the government has allowed £6 million (reducing to £5 million and £3 million in the two succeeding years).

Under the allocation scheme proposed by ABRC and accepted by the government, the medical and science research councils will benefit chiefly because they would otherwise have been "taxed" so as to free funds for other research councils having to close establishments. In reality, the Science and Engineering Research Council (SERC) will do best out of the new deal; its budget is due to increase from £279 million this year to £299.1 million (at 1984 prices) next year and to £306 million and £311 million in each of the two succeeding years. The Medical Research Council's budget will increase by £6 million between this year and next and then by a further £5 million in the two succeeding years. The budget of the Agricultural and Food Research Council is due to decline gently from next year, as forecast in previous forward looks.

Among the innovations of the new British science budget is a device whereby ABRC will keep up its sleeve (in the year beginning in April 1986) a contingency reserve, initially £6 million but doubling in the following year. At the same time, the cost of providing a secretariat for ABRC and of carrying out a few rudimentary

science policy studies will cease to be laundered through the budget of the Economic and Social Research Council, but will be identified separately.

The overall effect of the new proposals will on balance be neutral. Sir David Phillips, chairman of ABRC, says in the documents to be published this week that the extra money "will restore level funding". By the same test, the new arrangements will not make it possible to do much that is new. Nor will they help SERC and the British government to decide whether or not to pull out of CERN, the next big row in the research councils' calendar. □

EPA moves

Washington

PRESIDENT Reagan has named Lee M. Thomas to succeed William D. Ruckelshaus as administrator of the US Environmental Protection Agency (EPA).

Ruckelshaus, whose resignation takes effect on 5 January, has earned widespread praise for restoring morale at EPA following the scandals during the term of office of Anne M. Burford. Only one of the 20 political appointees who ran EPA under Burford remains today, the remainder having resigned, been fired or convicted of



Lee M. Thomas perjury. Thomas, a career bureaucrat, has been assistant administrator in charge of the "superfund" toxic waste programme.

Thomas, whose appointment has to be ratified by the Senate, is a political independent who seems to be acceptable to environmental groups. But with the President under strong pressure to find budget cuts, EPA must be a possible target. Richard Ayers, senior staff attorney with the Natural Resources Defense Council, wonders whether Thomas will carry the political weight to defend the agency, whose operating budget, at \$1,200 million, is still less than it was in 1980 in nominal dollars. Earlier this year, Thomas told the House of Representatives that a proposed budget increase for the superfund programme, tied to a new timetable, would actually slow down the superfund work.

Tim Beardsley

Natural environment

UK council counts pennies

THE British Natural Environment Research Council (NERC) acknowledged last week that it can no longer adequately support those who look to it for research funds. According to Mr Hugh Fish, the new chairman, NERC has been squeezed by the general decline of government support and by the unwillingness of government departments to commission research. Speaking at the launch of NERC's annual report, Mr Fish said the council would have to become more selective, and promised a "corporate plan" early in 1985 that might identify areas of research to be temporarily or permanently abandoned.

Anticipating the forthcoming corporate plan, Mr Fish thought there would be a bright future for deep seismic sounding, where grant applications are buoyant. By contrast, remote sensing has been disappointing. Dr John Bowman, NERC's secretary, said last week that the process of peer review may not be the most effective, but that NERC's activity in this field may be more pointed because of plans to work more closely with the Science and Engineering Research Council.

NERC's constitutional difficulty is the large proportion of its budget committed to its own institutions, which took two-thirds of what the council had to spend last year. By comparison, support for academic research last year amounted only to 17 per cent of what NERC spent. Mr Fish would like to see this proportion doubled, but he did not say by when.

As things are, in its support of terrestrial and aquatic life sciences, and of environmental studies in the physical sciences, NERC was able to meet less than a quarter by value of the grants applied for. Its report for last year records regret at not being able to support work in remote sensing of the sea surface, vegetation and soil hydrology and the development of geological ion probes. In short, NERC cuts the figure of a harassed housewife on a restricted house-keeping budget.

So why, for example, spend £10 million on support of the British Antarctic Survey? Mr Fish is glad that the British government has given this field priority and would like "similar treatment for other areas".

In one respect, however, NERC has been able to break new ground. The council has taken steps to market its services as a research organization willing to take on commissions, and has set up an office in Brussels to ensure that its constituent laboratories derive as much benefit as possible from the European Commission's budget. The report even offers the services of its Brussels office to British universities that might wish to make use of them — but at a fee.

Philip Campbell

US shuttle delay

More trouble with loose tiles

Washington

NEW technical problems with the heat insulation tiles of the space shuttle orbiter *Challenger* have forced the National Aeronautics and Space Administration (NASA) to postpone several scientific missions scheduled for next year. Worst-hit will be the Spacelab Earth Observation Mission (EOM-1), which will not now fly in July next year as had been hoped but will probably be merged with EOM-2 and flown in September 1986. EOM-1 consists largely of instruments that could not be used effectively on the November 1983 Spacelab 1 mission because of the delays to that flight. The Spacelab 2 and Spacelab 3 missions will also both be delayed by several months.

The latest delays will certainly increase the cost of keeping together the scientific groups in both Europe and Japan that have contributed experiments. The proposed rescheduling of EOM-1 was one of the topics discussed last week by Professor Reimar Lüst, director general of the European Space Agency, and NASA administrator James Beggs. The delays will also further tarnish NASA's image with the Department of Defense (DoD): a classified payload that had been scheduled for launch in *Challenger* last month has been shifted to January, when *Discovery* will be used instead. NASA is obliged to give DoD first priority on shuttle flights, with commercial payloads ranking second and scientific missions last.

The problems with *Challenger*'s heat insulation tiles have been caused by an unforeseen chemical reaction between a water-resistant spray used to protect the tiles from rain before launch and the adhesive holding the tiles to the orbiter's aluminium skin. New thicker tiles are now being fitted in critical areas using new techniques while other tiles are being replaced by an insulator fitted in blanket form. The modifications are likely to take at least a month, and *Discovery* will probably need similar attention next year.

NASA officials stressed last week that if, as seems likely, EOM-1 is delayed until the autumn of 1986, there will be some scientific benefits to compensate for the inconvenience and expense of waiting for an extra 14 months. Because the combined mission would not have to accommodate any other free-flying payloads, observation time should be close to the full 6 days' effective duration of the mission, in contrast with the 4 days of work that could be expected from a July 1985 flight. And, one official pointed out with engaging frankness, a combined mission will have a higher priority ranking than either would separately, and so is less likely to be affected by any future technical difficulties that cause flights to be rescheduled.

Although no final decisions have been

taken on which experiments would be used in a combined flight, there is no major problem of incompatibility of candidate instruments and it should not be necessary to drop any experiments. The major constraint will be the amount of time the orbiter must spend in different attitudes for different observations. The experiments that had been scheduled for a 1985 EOM-1 flight include several solar radiometers and spectrometers for which the shuttle cargo bay has to face directly towards the Sun, as well as an ultraviolet telescope to be pointed into deep space and a German/European Space Agency metric camera for Earth observation, both of which flew in Spacelab-1 but were not able to obtain all the desired data. The September launch date now being canvassed would allow growing-season observations of vegetation in Europe and is thought to be ideal for the metric camera. The add-on experiments that had been provisionally assigned to EOM-2 will observe the limb — the horizon as seen from space — and also require the cargo bay to be facing Earth.

The EOM-2 instruments are designed to

look at the effects of solar variability on the stratosphere, and in particular ozone concentration. One of these add-on experiments, called ATMOS (Atmosphere Trace Molecule Spectroscopy), will benefit from being in a 57° inclination orbit rather than the 28.5° orbit that had been planned for EOM-2, and the combined flight will offer the opportunity to compare the performance of instruments that would not otherwise fly together. Time competition from the solar observation experiments should not be a major difficulty, as all the solar observations can be completed in two 12-hour sessions.

There will, however, be real scientific losses for experiments that might have flown on both EOM flights, such as a Belgian grille spectrometer experiment that will be used for comparisons with results from ATMOS. Other instruments might have been flown in EOM-2 after modifications based on experience with EOM-1. Dr Doug Torr of Utah State University, who works on the Imaging Spectrometric Observatory (ISO), says that experiment was to have been used in different modes on the two flights and will have to be reconfigured. ISO, which obtains spectra of atmospheric airglow, could provide data on "shuttle glow" relevant to designing the planned US space station. **Tim Beardsley**

Royal Society

Dependant bites benefactor's hand

THE Royal Society threw its weight last week into the argument about the conduct of research policy in Britain, but in the measured language people expect of it. Like Sir Keith Joseph, the British government's Secretary of State for Education and Science, the Royal Society's president until next year, Sir Andrew Huxley, said last week (in his annual address) that he had been moved to worry about the condition of British academic science by a report on the proportion of good research applications being turned down by the research councils. The report, commissioned by the British Science and Engineering Research Council (SERC) was prepared by Sir Jack Lewis (Cambridge).

Everyone has his own idea of what is wrong. To Sir Andrew Huxley last week, it seemed important that support for basic science in Britain "is being milked" to support applied science which, in his opinion, should be the responsibility of the Department of Trade and Industry.

On CERN, the European high-energy physics laboratory at Geneva, and the question of whether Britain should continue as a member, Sir Andrew said that he could not understand why there is such a fuss when the cost of membership has fallen from 30 per cent of SERC's budget in 1978 to just over 20 per cent now. Referring in passing, and apparently dismissively, to the British government's recent proposal that there should be a European academy

named after Leonardo da Vinci, Huxley said that the benefit of that "would be trifling in comparison with the injury that would be inflicted by a body-blow such as Britain's withdrawal from CERN". Procedurally, Huxley does not want to see the British government pay for continued membership out of some other pocket than the science budget.

Perhaps the Royal Society's most timely disagreement with the British government was over the question of student maintenance grants, which are to be made overall less generous so as to increase the funds for academic research by some £24 million a year. Huxley said last week that he welcomed the result but "I deeply regret that these funds have been secured at the expense of students in higher education".

As a reflection of its discontent, the Royal Society seems to be planning a more deliberate, but still modest, increase of its support for studies of the present state of affairs. An inquiry into the "health of basic science" (which will concentrate on research, and the decline thereof, into solid state physics and chemistry and genetics) is being supported by two full-time research assistants. The society is also planning to recruit three professional people, and supporting staff, to keep an eye on the development of science policy in Britain. To ensure independence, the society will not use government money for the task.

John Maddox

Problems of defining length

SIR — Asher Peres¹ discusses three aspects of length measurement: the definition of the SI unit of length, the realization of that unit, and the use of the unit to perform a measurement, and touches on problems that we have already discussed^{2,3}.

The relevant postulates of Einstein's relativity theory have been established experimentally with an accuracy well beyond that of the present best methods of realizing the SI metre; particularly so for Michelson-Morley experiments⁴. The nature of length has not been detectably changed by the new definition, nor has the size of the SI unit. The Conference Générale des Poids et Mesures (CGPM) has not recommended any changes in the laws required for scaling up or down from the unit length in order to make length measurements, neither is one obliged to use light for this purpose. The unit is merely a reproducible scalar representation of the physical quantity against which other lengths may be compared. The only change is the manner in which the new definition is specified and realized. The velocity of light is not asserted to be constant, but if it is changing, then the size of at least one of the SI units will be changing also — relative to some absolute units to which we do not yet have access (the units of *gedanken* metrology). Thus for cosmological experiments, where the constancy of the unit is required over distances and times, comparable with the size and age of the universe, respectively, neither the SI units nor the natural units (e.g. where $e = m_e = c = 1$) can necessarily be relied on. The SI units are of a pragmatic nature, and are designed to provide the most invariant units that can be achieved with the science and technology of the day; if the present methods become detectably inadequate they will be replaced by something better.

The definition of a unit tells us little about the nature of the physical quantity concerned, and we need other instructions in order to know how to measure a physical quantity. The definition of the kilogram, for example, is clearly not intended to define the concept of mass, or to instruct on how masses should be intercompared. Similarly the definition of the metre is not intended to be a definition of what we mean by length, or of how lengths must be measured. The CGPM has redefined the SI unit of length, but not the meaning of length itself.

The CGPM provides a definition of the unit⁵ which gives sufficient information for the great majority of users. The definition is accompanied by a *mise en pratique* which contains information and recommendations concerning the manner by which the unit should be realized if the full accuracy is required. The onus is on any metrologist who chooses to use a different method to demonstrate that the unit so realized is of the same magnitude as that

produced by the recommended methods — moreover, it is stated that proper use must be made of the state of knowledge of the day in applying corrections for known effects (whether electromagnetic, quantum-electrodynamic or relativistic effects, and so on). Professor Peres is correct in stating that although the new definition might be thought to require two synchronized clocks, the practicality is that the round trip time is measured and divided by two. This is indeed the current practice in both interferometry and time of flight measurements (such as lunar ranging) and is implicit in the *mise en pratique*.

The CGPM recommendations are the result of extensive consultations with scientists and technologists throughout the world, but they cannot anticipate future discoveries other than by extrapolating existing trends. For example, if there were found to be small, but significant, anisotropy in the velocity of light (that is some preferred axis in space) then this property would either be incorporated in the definition itself or in the *mise en pratique*. At present we know that the new definition can be realized with a considerably greater accuracy than could be achieved with the former krypton-86

realization of the metre.

On the question of the use of the unit, Professor Peres uses light to measure the circumference of the Earth and so it follows that it must be in some form of waveguide in order to circumnavigate the Earth. In such a case it would, of course, be necessary to take account of a number of effects and to make the appropriate corrections, including that for the rotation of the Earth.

Relativistic effects can be encountered with measurements of mass, length, time, in electromagnetism and elsewhere, however their base units are defined. As accuracies improve, it will be necessary for more and more people to take these and other sophisticated effects into account before interpreting the results of measurements. It is certainly important to ensure that the units of experimental and theoretical sciences remain conceptually consistent whenever changes are made.

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English at risk

SIR — Your invitation (*Nature* **306**, 134; 1983) to readers for comments on the abuse of language seems to have evoked little response. May I reopen the issue?

Idso and Brazel (*Nature* **312**, 51; 1984) write: "Inclusion of this latter effect in a simple model of watershed runoff applied to 12 drainage basins in Arizona..." Various points made in the letter, and the North American addresses of the authors, suggest that by watershed they mean a catchment area.

So what distinction is being made in the quoted passage? Or is it just what Fowler and Fowler called "elegant variation"? I have already pointed out (*Nature* **307**, 312; 1984) that the meaning of watershed is so uncertain that it is unsuitable for use in the mixed company of an international readership.

A new verbal abuse is that the distinction between credibility and credence is in danger. A statement has credibility, credence is something given to it by a human mind. *Nature* is concerned with the written rather than the spoken word. May I however grumble about the error, often heard in radio science programmes, of pronouncing dissect as if were spelt disect. The word means "to cut apart" not "to cut in two", and one of the few almost unbroken rules of English spelling is that a double consonant shortens the preceding vowel.

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Exosat

SIR — Your news item "Britain in space — strong links with Europe urged" (8 November, p.92) refers to "some recent failures of instruments built for other people's satellites (such as some of the detectors for the Exosat satellite)". We should like to point out that the Exosat detectors which have failed were not built by a British space research group but by industrial contractors selected by the European Space Agency. The early development of the prototype detectors was carried out in this laboratory, but, contrary to previous practice, none of the flight hardware was built here. This point seems to have escaped many people's attention.

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Not our drugs

SIR — I write to draw attention to a substantial mistake in your leading article "What price drugs?" (15 November p. 184)).

The drugs Valium and Mogadon are produced by Roche Products Limited and not by Ciba-Geigy Pharmaceuticals; we are in no way involved with their production or marketing.

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Science and the philosophers

from George Gale

To scientists, the philosophy of science seems an irrelevance, as does the empirical practice of science to philosophers, preoccupied as they are with the logical consistency of their methods. The gulf between the philosophy of science, which has its roots in the growth of positivism in the late nineteenth century, impoverishes both. But there is now hope that the gulf will be bridged by the evolution of philosophy into theory of science.

The activities of the philosopher are simply irrelevant to my scientific life. This view, or one very similar to it, is most certainly held by a vast majority of practising scientists in the English-speaking world. It was not always so. Philosophy and the various sciences have long illustrated the intellectual analogy of the relation between parent and offspring, in which each new scientific discipline, in its turn, left its philosophical home in order to embark on its own independent career. Moreover, historical research clearly reveals that philosopher and scientist often cooperate closely during periods of conceptual crisis or revolution in the sciences in question, particularly when the issues involve methodological problems¹. For similar reasons, this pattern is also exhibited during the initial phases of newly developing sciences, as will be seen.

As might be expected, given a relationship of this sort, cooperation between philosopher and scientist lessens during periods of conceptual calm in a science, as the scientist engages himself in the everyday work of the laboratory and classroom. Physicist John Urry, among others, has suggested that present-day physicists' lack of interest in philosophical matters illustrates their contentment with the conceptual structure and methodology of their science as it is.

Yet the present-day scientist's disdainful attitude towards the activities of the philosopher goes far beyond what might be explained as the result of intellectual contentment. Something quite drastic has occurred, something which has severely weakened an interdisciplinary connection stretching back to the Greeks. In what follows, I attempt to uncover some of the elements involved, looking from the philosopher's side of the science-philosophy relationship.

Naturally enough, it is to be expected that analysis of such a fundamental issue would be quite a complicated effort, involving as it must discussions of recent historical developments in both science and philosophy; discussions about the struc-

ture and goals of contemporary higher education; and even discussions about practical features of the internal political structure of both professions. But despite the complexity and difficulty, such an effort clearly needs to be made if we are to understand the genesis of such a radically intensified view as that held by contemporary scientists in the English-speaking community. It is hoped that the discussion will serve as an initial effort to begin examination of this enormously significant topic.

Marriage and divorce

Philosophy and science emerged together, mixed and intermingled inchoately in the first attempts by classical Greek thinkers to understand nature. But even though they always remained linked in Classical thought, it did not take long for appropriate roles to be sorted out. Thus, already by the time of Aristotle, certain very definite demarcations had become apparent between the two disciplines. It is fascinating that, even today, when one reads any particular treatise of Aristotle's, it remains quite easy to see when he is thinking as a philosopher of science rather than as a practising scientist. Indeed, many topics originally developed by Aristotle continue to hold interest today. Thus it is that contemporary philosophers retain strong interest in both the logic of scientific thought and the structure of scientific method, two primary targets that were originally identified by Aristotle and his successors.

Strong interaction between philosophy and science is not, however, limited to the period of classical Greece. Right from the initial awakening in Copernicus, Kepler and Galileo, modern science and modern philosophy emerged together, to interact with one another in the production of a mature synthesis, a true world view, through the efforts of great philosopher/scientists such as Descartes, Leibniz and Newton². It is no accident that Newton thought of himself as a natural philosopher, that is, a philosopher of nature.

These examples demonstrate the closeness of the relationship between science and philosophy during the conceptual revolutions of the formative period of modern science. Scientists and philosophers both believed strongly that the two disciplines are inextricably linked in the general structure of human knowledge itself. For this reason, they believed that interaction was not only beneficial but necessary to the ultimate success of human inquiry into nature. Indeed, Descartes, Leibniz and Newton believed so strongly in this connection that they took it for granted and worked actively in both disciplines. Later, even after scientific professionalization had begun to decrease somewhat the number of scientist-philosophers, philosophers and philosophy itself remained in close and beneficial communication with the sciences. Immanuel Kant, the great eighteenth century German philosopher, in fact regarded providing the philosophical foundations for classical physics to be his most significant task³. His efforts endured for nearly a century as hallmarks of the science-philosophy connection.

After Kant, the eras closer to our own time continued to exhibit the connection between science and philosophy. In biology, Darwin and the philosopher Whewell argued long, hard and successfully in critical analysis of the conceptual and methodological content of the revolutionary theory of evolution. Later, when early twentieth century physics experienced what can only be called revolutionary upheavals, as the theory of relativity and quantum physics overthrew the classical world view, the classical philosophy developed by Descartes, Leibniz, Newton and Kant was overthrown as well. Indeed, it can be argued convincingly that the philosophical consequences of these two new physical theories were at least as important as the theories themselves.

Such would most certainly be the view of many of the participants in the revolution; Bohr, Einstein, Planck, Heisenberg, DeBroglie among many others⁴. Thus, it

would seem that at least in that most fundamental of the sciences, physics, communication and interaction between philosophy and science continued unabated at least until late in the third decade of this present century. (Indeed, a small community of scientist-philosopher collaborators continues to work in both quantum mechanics and relativity theory, an obvious exception to the general situation.)

In the face of such a vigorous historical tradition, today's perceived irrelevance of philosophy to science stands out starkly. One can only ask, "What in the world happened?" Initial answers to this question must focus first upon internal topical developments in recent philosophy.

Reforming science

The ultimate irony of the present divergence between scientists and the philosophers is that its roots lie in the efforts of a philosopher-scientist to bring about a philosophical reform in physics. Ernest Mach, the philosopher-scientist in question, believed that physics — and the natural sciences in general — had become seriously infected by the post-Kantian metaphysical idealism that dominated the faculties of major German universities. In particular, he argued that the easy acceptance of hypothetical concepts, concepts which typically referred to unobservable theoretical entities such as energy, force and absolute space and time, had proliferated to the point where physics was populated by an incredible metaphysical bestiary.

Mach sought to reform physics entirely, to chop, prune and pare its theoretical structure until only what was "positive science" remained. From this stated desire came the general rubric for the ensuing reform. Following the notions originated by Comte, Mach and his successors took up the movement called "positivism"; and while there are other important reasons behind the present disaffection between science and the philosophers, those reasons which are substantive, and rely upon topical disciplinary content, lie mostly in the beliefs of positivism and their effect upon several generations of philosophers in the English-speaking world.

Positivism has three tenets, each of which entered the doctrine at a different time, under the aegis of a different individual⁵. Mach, in 1886, provided the "empiricist criterion of meaning"⁶. Poincaré (1902) suggested the "conventional view of mathematics"⁷. Whitehead and Russell (1910–1913) developed "the axiomatic system for symbolic logic"⁸. And finally, in 1923, Rudolf Carnap introduced a complicated synthesis of the three elements in what can be clearly recognized as what we today call "positivism"⁹. (The movement is also variously called "logical positivism" and "logical empiricism" in obvious reference to alternative emphases on various of the

major principles.)

Each of the three principles contributes something distinct to the positivist theory about scientific theory. Mach's empiricist criterion of meaning represents his attempt to eliminate reference to unobservable entities from science. Roughly stated, the criterion disallows from acceptance into legitimate science all terms that do not have an empirically observable entity, or event, as a referent. Since, for example, according to Mach, the term "absolute space" does not name an object or event which is observable in the physical/empirical world, absolute space must not be allowed as a concept in legitimate science. In this fashion, both observational terms and observation itself became the essence of science. Bridgeman's operationalism and Popper's falsificationism are obvious inheritors of this tradition.

Unfortunately, one initial logical consequence of Mach's empiricism was the required elimination of mathematical terms and operations from properly cleansed and thus legitimized theories. This grotesque result follows from the fact that purely mathematical concepts have no observable referents; for example, "plus", "sum" and "differential" are not the proper names of any empirically observable physical object or event. Further, since the empiricist criterion of meaning also laid down the rule for which sentences could qualify as being true or false, all mathematical sentences automatically became meaningless. After all, if theoretical terms are meaningful only if they have observable referents, then all mathematical terms are meaningless precisely because they lack such referents. It follows directly that mathematical sentences, which contain only meaningless terms, must be themselves meaningless, and cannot even be candidates for truth or falsity.

Maths and truth

Recognizing the unsatisfactoriness of this bizarre result, Henri Poincaré found a way minimally to preserve mathematics and yet hold to the empiricist criterion at the same time. His proposal, stated roughly, was that mathematics, while it is not strictly speaking empirical, is, even so, associated with the conventional activities of scientists. That is, even if mathematical statements cannot be candidates for truth or falsity, at least they are not meaningless, since they capture meaning from the conventional practices of theoretical scientists. Moreover, according to Poincaré, if necessary, mathematical sentences are ultimately eliminable from science, since their substantive content is reducible to strings of sentences containing only empirically meaningful observational terms. On this view, it is clear that the formalisms of scientific theory itself are, strictly speaking, redundant, since they represent only the conventional way scientists speak about what, in the end, reduces to strings

of observation terms. After Poincaré, positivism lacked only a suitably precise language in which to carry out the proposed reform of physics. That language was provided by the achievement of an axiomatic system for symbolic logic.

Philosophical concern to impose logical methods on scientific thinking is an ancient and honourable endeavour. It was Aristotle himself who laid it down that deductive logical structure is a necessary condition for any discipline to call itself "scientific". This demand was scarcely relaxed during the development of the modern philosophy and science, as Descartes and Leibniz clearly evidence. But what had always led to failure in previous attempts to make science logical was the lack of a sufficiently rich, powerful and precise logical apparatus. Deductive syllogisms produced from the syntax and semantics of everyday speech just could not do the trick. Whitehead and Russell's axiomatic system for symbolic logic, the logic of quantifiers and predicates with identity, made available for the first time a language which seemed to offer the potential to capture significant aspects of scientific thoughts. Unfortunately, as was later revealed, logicistic efforts cause more problems than they solved. But, at first, optimism reigned.

Rudolf Carnap produced the first mature interpretation of logical positivism, a synthesis in which the three original principles were the foundation. According to this view, legitimate scientific theories were to be constructed along the following lines. At the foundation level, terms were to be introduced in strict association with observation, and put into definitions stated in the predicate calculus. "Space", for example, would be associated with "measurements made with a ruler", "time" with "measurements made with a clock" and so on.

It was even hoped that complex predicates such as "soluble" and "homeostatic" would succumb to this analysis. Once these terms were safely stated in the new symbolic logical system, they would become amenable to manipulation by logical operations and accepted mathematical functions. Theoretical terms would emerge from these operations. "Velocity", for example, could be introduced as the "dividend" of 'space divided by time', and "acceleration" could then appear as a further dividend. In this stepwise process, all theoretical terms could be examined, analysed and ultimately rejected if they failed logical derivation of this sort.

Carnap's programme was quickly hailed as the first successful candidate to fulfill the philosophers' millennial-old desire to apply their distinctive technique of deductive logical analysis directly to scientific thinking. But terrible problems immediately surfaced. For the most part, these were intrinsic to the positivist programme itself.

Cosmologist Brandon Carter has

recently noted one particularly prickly issue. Even given Poincaré's conventionalist interpretation, accepting the empiricist criterion of meaning causes problems for those whose scientific activities most intimately involve mathematics, the theoretical physicists. In the first place, Carter notes, theoretical physicists are most uncomfortable with the notion that mathematical results might be meaningful (in a certain limited sense), but could not be candidates for falsity or truth. Moreover, he believes that most theoretical physicists would in nearly all cases feel much less confidence in assigning truth or falsity to sentences employing empirical observation terms than to mathematical sentences. As Carter remarks, he is much more comfortable trying to decide whether a mathematical sentence is true than he would be faced with such a decision regarding a statement about the physical world.

The response of many philosophers to Carter's view would be revealing. "Carter does not understand the nature either of truth or of mathematical systems", they would claim. Many would argue that his view is naive, indicating a need of education in modern philosophical theories. Such a response is clear evidence of the breakdown in communication between philosophy and the scientists. Regardless of the correctness of either side, it is clear that neither is attending properly to the other.

Reform

One aspect of this situation is especially ironic. A philosophical reform movement instituted by a theoretical physicist specifically to cleanse his own discipline philosophically, has succeeded in alienating a sizeable proportion of the practitioners of that very craft from an interest in either philosophers or philosophical matters.

Several further points must be mentioned. First, although some attempts have been made to extend positivist programmes beyond physics (one might think here of behaviourist psychology, which began life as a discipline specifically constructed according to the guidelines of positivism), for the most part, positivist philosophers have focused on physics. Such a limited perspective on science has of course limited the range of relevance of recent philosophy of science. Given the developments in many sciences over the past sixty years, this restricted focus has isolated philosophers from a vast number of new and/or improved sciences.

A second problem involves the positivist language itself. Modern symbolic logic has itself matured into a discipline with a highly developed system of techniques and processes. In order even cursorily to follow relevant discussions by modern logically-minded philosophers, scientists must become fluent in the symbolic language. Since they cannot be expected to do so, it becomes incumbent upon philosophers

themselves to initiate and maintain communication and interaction. But there is little evidence that this has happened.

In fact, it appears that interest in logical analysis and other formal questions has quite isolated a sizeable segment of the philosophical community, keeping them from being concerned with issues that concern their scientific colleagues. As physicist James Cushing has noted, this sort of interest has introduced issues into the philosophy of science that do not occur in actual science. Thus, scientific questions which might once have been of interest no longer attract philosophers, in part because they lie outside current philosophical practice.

Accordingly, if a scientific issue does not involve formalizable questions, then it does not concern logic-oriented philosophers. One example is the stance of philosophers with regard to the anthropic principle, a significant topic in cosmology for almost fifteen years. This principle raises both methodological and substantive questions in that it claims that the existence of human beings is not a neutral fact in the Universe but that our existence, by itself alone, has implications for what may be observed — and theorized — in cosmology¹⁰. Issues such as this should be of obvious interest both to philosophers generally engaged in traditional enquiry about deep human concerns and also to those whose focus is more narrowly on the analysis of scientific methodology. Yet little interest has surface about the first concern and essentially none about the second.

Indeed, when I recently discussed the topic in print, almost all the letters and comments I received from philosophers were abusive¹¹. The comment "You have set philosophy back to the time of Copernicus" is typical. I was asked why I "even bothered to interest myself in such an issue". My only reply, then as now, was to note that any scientific development as significant as the use of anthropic arguments represents not only a *legitimate* topic for philosophers, but also a topic that is important, given that so many excellent scientists occupy themselves with it. Not to take a professional interest represents, again, the growing isolation of philosophy from science.

One final factor is perhaps the most important, given the growing professionalization of philosophy and especially of the philosophy of science. As it turns out, the application of the logical calculus to scientific thinking contains its own insidious problems. Since in any general case, interpretive use of a theoretical system imparts into the resulting interpretation flaws endemic to the theoretical system itself, it is not odd that this kind of interpretive use of the logical calculus has often revealed weaknesses not of scientific thinking, but of logical theory. Thus, in attempts to interpret the methods by which scientists set about confirming,

verifying or accepting hypotheses, formal problems intrinsic to the logic of the conditional "if, then" operation, which must be used in modelling scientific prediction, soon turned philosophers away from positive accounts of verification through prediction. Instead, falsification, *à la* Sir Karl Popper, was proffered as the proper legitimate mode of assessing scientific theory, for no other reason than that its logic is much cleaner than that of prediction¹². According to the falsificationist model, scientists' ultimate task is to falsify and discard theories rather than to confirm and accept them. Regardless of whatever other virtues there might be in the falsification programme, and Popper's programme especially, such a conception of the ultimate scientific task is bound to seem odd to thinkers who believe their scientific lives to involve a search for truths about nature.

But even more important is what has happened to professional philosophers as a result of the intricacies of the logical calculus they have adopted as an instrument. In effect, philosophers have been enticed away from the problems of interpreting science in order to focus on problems internal to the means of interpretation rather than the substance. A survey of the philosophical literature during the 1940s, 1950s and 1960s reveals a proliferation of articles devoted to complicated and technical questions of a logical nature which, although derivatively related to the analysis of science, are most conspicuous for their primary concern for the details of the logical calculus, not the details of the science. These almost arcane discussions, verging on the scholastic, could have interested only the smallest number of practising scientists.

Coinciding with this inward-turning of perspective, academic philosophy in both Britain and the United States experienced the greatest period of growth in its history. Growth of philosophy faculties entailed expansion of graduate student ranks, which naturally made it necessary to find tasks on which to engage the new philosophy candidates. Happily enough, the internal troubles of the logical model for science were severe enough to absorb even this new flood of workers. So the logical positivist theory became doctrine, with a vast new influx of defenders and practitioners. Among philosophers who now professionally interest themselves in science, a graduate student background in positivism predominates. And although it would be unfair to say that the formalistic issues of the logical positivist programme still represent the doctrinal concern of philosophy of science in general, there persists a major thrust in that direction. Many philosophers would agree with the leading protagonist of this effort who recently told me that scientific thought is philosophically interesting only to the extent that it is formalizable.

The upshot of all this is that, because of

its substantive content, the past two generations of philosophical concern with scientific thought have increasingly driven the philosophers away from their traditional interaction with science in general, have restricted philosophical focus to a narrow range of physical theorizing and, finally, have turned the gaze of philosophers away from science itself and towards the internal deficiencies of the means of analysis, symbolic logic. This movement has had disastrous effects on communication between philosopher and scientist. Unfortunately, aiding and abetting these internal topical developments in philosophy, there has been a series of developments in the academic politics of both philosophy and higher education in general which has exacerbated the crisis of divergence.

The wider context

Accompanying the growth of philosophy faculties in both Britain and the United States was a concomitant growth in professionalization, especially noticeable among the ranks of the philosophers of science. Indeed, it is not outrageously unfair to say that philosophy of science came into existence as a profession, separate from philosophy itself, during this period.

To a certain extent, this professionalization of the philosophy of science required the emergence of an identifiable set of commitments and beliefs that could function as professionally unifying tenets. Logical positivism satisfied this function. Although not all philosophers of science identified themselves as positivists, positivism provided the standard against which to define themselves, positively or negatively. And the positivist tenor of the times was inescapable, even for those who foreswore the doctrine itself.

All the institutional hallmarks of professionalism accompanied the doctrinal unification. Membership in professional societies grew and, in turn, the various activities typical of professional societies strengthened the profession. Conventions, professional colloquia, the publication of journals and the like cemented the membership together, providing a sense of purpose and, in general, reaffirming the substantive movement of the times. The ultimate institutional outcome of this professionalization showed itself in two ways. "Philosophy of science" positions were provided in nearly all sufficiently large departments of philosophy while departments devoted solely to philosophy (and latterly, history) of science were instituted at a number of important institutions. But contrary to what might have been expected, institutionalization of the profession raised problems for the philosophy/science connection.

To keep an academic department healthy, especially one as specialized as in philosophy and history of science, a certain mass is necessary to ensure direct interac-

tion and cross-fertilization. When a department is too small, healthy diversity of opinion, tolerance for other views and intradepartmental learning, seems never to arise. In short, departments fail to grow in perspective and knowledge, and one particular viewpoint tends to dominate.

Problems of critical mass have always plagued the specialized philosophy of science groups in Britain, which could certainly explain the apparently monolithic doctrinal nature of British philosophy of science. With budgets short, the prospects for improvement are not bright.

A likely consequence of this unhealthy situation is stagnation in philosophical research, and an attendant inability to turn away from internal institutional problems towards interaction with scientists. With the stance of philosophy already directing energy from direct involvement with genuine scientific practice, the ultimate results are likely to be disastrous.

A second quite different problem, concerns the internal politics of philosophy departments. In the recent past, most Anglo-American philosophy departments have taken on an analytic cast, with "Wittgensteinian" departments (and there are many) representing the most extreme cases. Philosophers of science, even where special philosophy of science sections are integrated into the general departmental structure, are always in the minority. They are thus subject to any consequence that may derive from the way in which the nature of their professional interest conflicts with the analytic nature of the greater department. To put it bluntly, conflict appears to be inevitable.

Contemporary analytic philosophy often expresses a strong mistrust, indeed hostility, towards the empirical world and to those who even give the appearance of dealing with it. Views such as this are not new, but are familiar from the time of Plato. But in its modern guise, analytic philosophy has always emphasized the limitation of its interest to the conceptual aspects of human experience, eschewing concern for the empirical world of nature.

The political repercussions of this stance were not long delayed in the newly-professionalized philosophy of science. Insofar as a person's work exhibited elements that might be construed to be "empirical", his work was thereby held by the majority to be "non-philosophical". Moreover, contact with real practising scientists was at least questionable from the point of view of authentic philosophy. Since departmental political power typically rests in the hands of others than philosophers of science, conflicts such as this raise problems not only for philosophers of science in general but, particularly for those who might have interests in close engagements with individual scientists or empirical scientific practice, both of which require of philosophers extensive immersion in the empirical content of the target scientific disciplines.

The consequences of these political problems are all too familiar. In one case, for example, hostility from a strongly analytic department has recently forced a well-known empirically-minded philosopher of science to change institutions. In another case, a philosopher in a large British philosophy/philosophy of science department has been the target of hostility from the majority of other philosophers, simply because he has been congenial to members of the philosophy of science group. One need not even ask about the status, in this department, of the philosophers of science themselves.

In other cases, philosophers of science who tackle empirical problems, who appear on programmes of scientific meetings or who publish in scientific journals have difficulty convincing their departments, or the various academic review boards, that their work is proper "professional" philosophy. When competition for salary, tenure and recognition is intense, such problems are of great practical importance. And even if these are isolated cases, and not withstanding this intradepartmental hostility in every kind of department, philosophy or other, it is clear from the style and tone of the cases which have arisen that there is specific animus mitigating strongly against contemporary philosophers of science who would initiate the first difficult moves to re-establish their part of the traditional link with the community of scientists.

One idiosyncratic feature of the United States is that component of American mythology, which has it that the philosopher is the quintessential theorist, the paradigm of the deep thinker whose head-in-the-clouds activities render him irrelevant to the practical world. Given this long-standing attitude, it is mystifying that US universities even bother to maintain departments of philosophy. But in the recent vigorous tailoring of US university education to the requirements of professions and the workplace, perceptions of the relevance of philosophy have further diminished as courses have been dropped, programmes curtailed and the number of majors have plummeted.

Naturally enough, philosophy of science is tarred with exactly the same brush, the internal divisions in philosophy departments notwithstanding. The academic community's growing perception of the irrelevance of the philosopher of science *qua* philosopher, when coupled with the restrictive concerns of the discipline itself, make it almost inevitable that contemporary science students in the United States will not only develop in an environment where philosophy is taken to be irrelevant, but will have only a slim chance of encountering those who might demonstrate otherwise. So stands the situation today. Fortunately, there are some signs that, at least in some areas, further divergence between philosophy and science is not unavoidable.

A brighter future?

Several recent developments suggest at least a hope of some improvement in the relationship between science and philosophy which involve, on the one hand, some of the newer sciences as well as theoretical biology and, on the other, doctrinal changes in philosophy of science itself.

Clark Glymour has recently noted that newly emerging sciences tend to provide considerably more opportunity for relationships between philosophers and scientists. He pointed to computer science and, in particular, artificial intelligence, where there has been close collaboration between scientists and philosophers. Interestingly enough, this relationship extends even into substantive matters; one recent expert system, a medical diagnosis routine, was designed from the outset to embody a falsification testing strategy. It will be more than slightly ironic if the logical formalization programme then turns out to provide an acceptable basis for artificial scientific intelligence when it has not been accepted as a basis for the more natural versions of scientific intelligence.

Contemporary biology is another field of increasing interaction between scientists and philosophers. Whereas twenty-five years ago, respectable biologists would have had little or no interest in philosophical issues, contemporary biologists and philosophers are engaged in a surprisingly vigorous interaction with one another. Marjorie Grene, whose philosophical encounters with biology bear witness to the vast changes during this long period, believes that two factors are involved. First, biologists themselves have suffered such sweeping, indeed revolutionary, changes in both concepts and methodology that they are necessarily much more open to philosophical concerns. Moreover, perhaps more important, there is now a generation of biologically well-trained philosophers, whose strong grounding in the empirical details of the science commands the respect of biologists. It is interesting that, as Grene and Glymour note, many of those belonging to the vigorous core of contemporary philosophy of biology were partly trained in Harvard's interdisciplinary programme.

Others have noted that the philosophy of biology has never developed a strong doctrinal allegiance with logical positivism and, for this reason, has not been isolated from its subject as were the more physically oriented philosophers of science.

Radical changes in the substance of philosophy of science also offer some hope. Everywhere in the English-speaking world, but especially in the United States, where the influence of falsificationist

doctrine was never so monopolizing as in Britain, philosophy of science is undergoing a radical shift of methodological orientation, away from the formal and logical towards the empirical and historical. This change has been brought about by the challenges to positivist doctrine embodied in the works of Thomas Kuhn¹³, Norwood Russell Hanson¹⁴ and others.

Some of the issues raised by this revolution go directly to the foundations of the philosophy of science. Thus several philosophers of science, most notably Giere¹⁵, have challenged philosophers and philosophers of science alike with the idea that philosophy of science may itself be an empirical and essentially scientific activity, not the *a priori* conceptual discipline exemplified in traditional departments. Consequentially, there have even been suggestions that this more historical/empirical philosophy of science would be more aptly named "theory of science".

Developments such as these, unfortunately, can only exacerbate the hostility of many traditional philosophers, and that bodes ill, at least in the short run, for the philosophers of science, politically isolated as they already are in their academic department of philosophy. But in the long run, the situation of the philosophers of science may be bettered from a surprising direction — the sciences themselves. A growing emphasis on historical and empirical components in philosophy of science would tend to bring about closer relations between scientists and philosophers, and this for several reasons.

The shift away from the arcana of technical logic towards the language of historical and empirical analysis will facilitate communication between the two disciplines, when scientists most surely will regard philosophers as methodologically and linguistically less alien. Second, it is possible that the subject matter of an historical/empirical philosophy of science would be intrinsically more appealing to scientists; certainly, it is always more interesting to find one's own behaviour and methods described rather than prescribed, as under the earlier positivist regime.

Schism

Finally, as philosophy of science more and more evolves into theory of science, certain very practical effects may compensate for the losses brought about by the schism between the new theorist and his erstwhile base in traditional philosophy. As Henry Frankel has remarked, under such conditions, many elements of the established reward structure in science would be open to the historical/empirical theorist of science. For example, Frankel notes, grants from the US National Science

Foundation might become accessible; Kristen Shrader-Frechette has noted that administrators at US universities are especially appreciative of grant receivers, and doubly so if they are engaged in interdisciplinary activities.

Naturally, it is by no means certain that the possibilities suggested by Giere and others are likely to occur. But if it is considered desirable to establish an essential interaction between two fields which, by both nature and history, should be strongly interacting, fostering the evolution of philosophy of science towards theory of science would be one excellent strategy.

A quite fascinating speculation arises from this possibility. The history of modern science is the history of one field after another moving out from under the sheltering aegis of philosophy, in order to establish independent dominion over a distinguishable empirical territory. Thus emerged cosmology, physics, chemistry, biology and the rest. Were theory of science to emerge as suggested, would it not be none other than the most recent emigre in this long succession? That question raises exciting issues reaching to the foundations of human knowledge, issues that must needs be answered. It is time to begin.

Some of the historical material herein was presented at an American Association for the Advancement of Science Symposium, 21 June 1983, at Logan, Utah. I have benefited immensely from conversations and correspondence with many people, including B. Carter, J. Cushing, H. Frankel, R. Giere, C. Glymour, M. Grene, R. Harré, M. Rees, G. Ross, K. Shrader-Frechette, J. Urani, J. Worrall. The work was supported in part by a Weldon Spring Grant, and the University of Missouri — Kansas City. □

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Similarities among retrovirus proteins

SIR — In recent correspondence to *Nature*, Cianciolo *et al.*¹ reported a region of similarity between the transmembrane protein p15E of murine and feline leukaemia viruses and gp21 of the human T-cell leukaemia virus types I and II (HTLV-I and -II). We agree that these proteins are indeed similar and draw attention to additional sequence similarities and structural features common to the transmembrane proteins. The transmembrane proteins of Rous sarcoma virus (RSV), bovine leukaemia virus (BLV) and mouse mammary tumour virus (MMTV) are included in the analysis.

Figure 1 shows a schematic comparison of the transmembrane proteins and Fig. 2 depicts three regions of amino acid sequence similarity (regions IA, IB and IC). The following features are present in all of the transmembrane proteins considered:

The amino-terminal domain of the transmembrane proteins has a hydrophobic character and contains region IA. This domain is followed by a strong polar region. The position of the hydrophobic region at the amino terminus of the transmembrane protein is reminiscent of the fusion sequence of other viral transmembrane proteins such as the Sendai and influenza viruses (see ref. 2 for review). For this reason, we speculate that this region of the HTLV envelope may be responsible for the fusion activity observed for HTLV virus³. The spacing of cysteine residues located near the middle of the transmembrane proteins is also conserved. Moreover, these residues occur in a highly conserved amino acid sequence context

(region IB). We postulate that this region is in direct contact with the exterior glycoprotein, and that disulphide bonds are formed between these two cysteine residues and the similarly spaced and conserved cysteine residues close to the middle of the external glycoproteins. Conservation of sequence in this region might be dictated by structural constraints required for protein-protein contact. Potential glycosylation sites are located near the carboxyl end of region IB in most of the viruses considered. Glycosylation can occur at these sites, as the transmembrane proteins of HTLV-I, HTLV-II, BLV and MMTV are glycoproteins.

There is a hydrophobic region near the carboxyl terminus which includes a highly

conserved sequence (region IC). This region has the correct length and position to traverse the cellular membrane. An α -helix is the most favoured configuration of this portion of the transmembrane region^{4,5}. Moreover, this hydrophobic domain terminates with a positively charged amino acid located three residues (except in RSV) carboxyl terminal to a conserved cysteine residue.

A small, highly variable region is coded for by the extreme carboxyl terminus of the envelope gene precursor of retroviruses. This region, designated R, is cleaved from the envelope gene precursor late in maturation in murine viruses⁶.

At present, there is no experimental evidence to discard the involvement of any of the regions of similarity or structural features reported in the immunosuppressive activity demonstrated for murine and feline p15E.

A more extensive analysis of similarities among the p15E as well as other retrovirus proteins is contained in our review of HTLV-I structure³.

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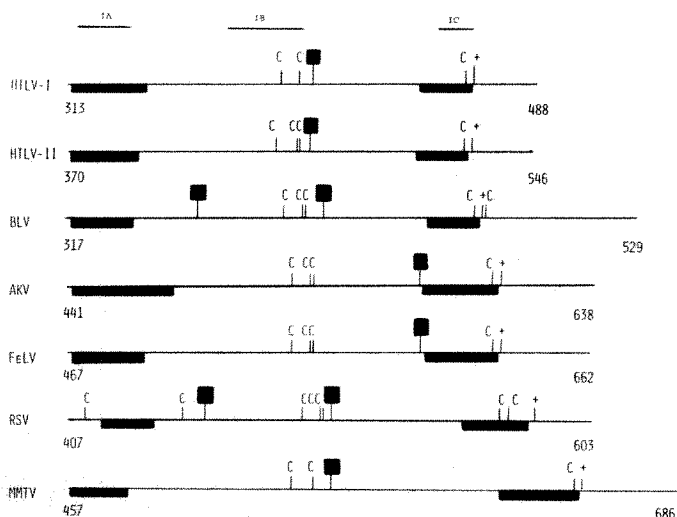


Fig. 1 Schematic comparison of the transmembrane proteins of: human T-cell leukaemia virus types I (ref. 7) and II (ref. 8) (HTLV-I and -II), bovine leukaemia virus⁹ (BLV), AKV⁴, feline leukaemia virus¹⁰ (FeLV), Rous sarcoma virus¹¹ (RSV) and mouse mammary tumour virus¹² (MMTV). The hydrophobicity pattern was obtained using the Intelligenetics PEP program. The hydrophobic regions discussed in the text are indicated by a thick bar beneath the line. Potential glycosylation sites are indicated by a square, cysteine residues by a C and the position of a positively charged amino acid 3' to the transmembrane region is depicted by +. The position of regions IA, IB and IC from Fig. 2 is also shown for HTLV-I.

REGION	VIRUS	POSITION	SEQUENCE
IA	HTLV-I	315	PVAIVWLVSA
	HTLV-II	372	PIAVWLVSA
	BLV	317	PVAALTLGLALS
	AKV	439	REPVSILTALL
	FeLV	465	REPISLTVALML
	RSV	423	PTARIFASILAPG
IB	HTLV-I	377	QNRRLDLEWEQ
	HTLV-II	433	QNRRLDLFWEQ
	BLV	380	QNRRLDWYIRL
	AKV	509	QNRRLDLFLKEG
	FeLV	535	QNRRLDLFLQEG
	RSV	479	QNRRAIDFLLAN
IC	HTLV-I	452	LLLLVILAGPCIL
	HTLV-II	508	LLLLVILFPGCIL
	BLV	460	ALFLLFLAEPCLL
	AKV	590	ILLILLFPGCIL
	FeLV	616	ILLILLFPGCIL
	RSV	564	LLLLVCLPCLL
MMTV		630	ALLLVILMIFPL

Fig. 2 Alignment of three regions of sequence similarity among the transmembrane proteins of the retroviruses mentioned in Fig. 1. Conserved positions are boxed and conservative substitutions underlined. Although MMTV does not show significant similarity in regions IA and IB, it was included because the general structural features described in the text also apply.

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Privacy and the peer-review system

There are disturbing signs that the peer-review system for unpublished manuscripts is no longer as secure as it should be. Commercial competition is not the most serious a cause of trouble.

MOST complaints about the peer-review system say that it is unjust. But what about the possibility that it has become unworkable? A week ago, a patent attorney wrote on behalf of an author, whose manuscript could not be published in *Nature*, asking whether referees are required to sign a "confidentiality agreement" in advance of being asked to look at authors' manuscripts. His letter went on to allege that the existence of his client's manuscript, and perhaps even its content, had been disclosed to a graduate student at a university in the United States by a member of the faculty, who claimed to have been one of the referees (which is true).

Coincidentally, a colleague of another author (whose manuscript has also been refused) telephoned to say that one of the audience at a recent meeting had chipped in, at the end of a brief formal presentation of the still unpublished work, with a detailed critique based on a sight of the unpublished manuscript, said to be "all over the scientific community". That claim is probably exaggerated; probably the questioner meant "all over my laboratory" (where one of the referees also works). But whatever the truth, the tale is evidence that the peer-review system, whatever its other weaknesses, is not always the confidential means by which colleagues are consulted about the merits of each other's work.

To many people, the cynical and the embittered for obvious reasons, this discovery will not be a surprise. Why, they will say, should scientists who are good enough to help journals (usually without material reward) to decide what to publish also keep their own counsel about the manuscripts that pour endlessly across their desks? And now, when so much of academic science has an obvious commercial value, is it not inevitable that somebody who is actively working in a particular field will make the most of a scientific manuscript sent for review?

There is no denying that access to an unpublished manuscript can often be of great value to a referee. There is a host of fields in which this is the case — the development of semiconductor devices, solid-phase catalysts and photovoltaic devices driven by solar energy, for example. But the competition is especially fierce in the applications of molecular biology to biotechnology where the knowledge of even a short nucleotide sequence of a gene may, for example, enable a competitor to construct the nucleotide probe with whose

help the intact gene may be fished out of a DNA library. That, legend has it, is why people speaking on these topics at meetings in advance of publication tend to show their slides of nucleotide sequences for only very brief intervals — and why there has emerged a group of auditors skilled at memorizing a whole string of nucleotides seen only for a few seconds.

So the incentives for referees to break confidence are considerable. Authors are often painfully aware of the dangers, sometimes to the point of seeming paranoid on the subject. An author's recent claim that a recent piece of published work (which turned out to be mistaken) was being replicated at two other laboratories within a week or so of the submission of the original article was found, on investigation, to be only half-true; the work had been begun before, and was independent. The author had, however, guessed the geographical location of two of the three referees.

These are not the only hazards of the peer-review process, as exemplified by a sad tale from earlier this year. A manuscript reporting part of an incomplete amino acid sequence was in part concerned with what seemed an interesting homology with another amino acid sequence, sent for review to one whose interests include the maintenance of a databank. In the event, the referee spotted a more complete and striking homology than the author had claimed. That information was conveyed to the author together with a tactless suggestion (which was misunderstood) that there should be some formal recognition of what the referee had done. The upshot was that the article was withdrawn (fortunately to be published elsewhere) and the offended author convinced, as he explained, that he "had been robbed of the joy of discovery".

So should referees also take care not to let their intellectual curiosity run riot over papers sent to them for review? Of course not. All journals know of papers that have been enormously improved by the contributions made by referees. Sometimes, authors are ready to acknowledge this, although it must be rare that a referee's contribution is so great that the interpretation of data is entirely transformed. But these, in any case, are not the occasions when authors most fear that their data will be misused before publication.

The problems recently encountered with manuscripts whose content may have

immediate practical value in, for example, biotechnology are much more taxing. The obvious danger is that improper commercial use will be made of data that are still confidential, which is why journals try to use their common sense and not send papers for review to people likely to have a commercial interest in them. But there are two circumstances that make this ideal in general unattainable. First, and quite deliberately, the larger biotechnology companies have made a point of keeping close links with academic science, partly because the step between discovery and practical innovation is so short and partly because it helps in the recruitment of able people. Second, and much more serious, this is now one of many fields of science in which there are very few able academic researchers who do not have links of some kind with some commercial organization, commonly undisclosed.

So has the time come to recognize that the peer-review system has broken down? Again, the answer is flatly no. The interest of patent attorneys suggests that most authors are now keenly aware that the work they seek to report may have commercial value, and that they know what steps must be taken to protect it. Even where research has no immediate application, journals such as this do their best not to send papers for review to groups known to be in competition, or willingly follow an author's request that the referees should not include people working in named laboratories. (Nervous authors would be comforted to know how often referees disqualify themselves on the grounds that they have too direct an interest.)

There is, however, unsystematic evidence, chiefly what seems to be a greater volume of complaint, that some referees are now less than strictly bound by the request that they should deal confidentially not merely with the content of the manuscripts they are sent but even with the fact that they exist. The damage done by such transgressions is intangible but no less worrying on that account. The chief casualty is the civility of academic life. Authors are entitled to expect that their papers will be dealt with privately. Not merely their self-esteem but their public reputation may easily be damaged unfairly by gossip, necessarily incomplete, about the ways in which their latest attempts at publication have been handled. Erring referees should reflect that they are usually authors as well.

John Maddox

Sensory and energy transduction

Light-activated retinal proteins

from Lubert Stryer

THE discovery of bacteriorhodopsin (BR), a light-driven proton pump found in halobacteria, revealed that the photoisomerization of retinal has a significance extending beyond its role in vision in higher organisms¹. Two more light-activated retinal proteins have recently been identified in halobacteria: halorhodopsin (HR), which serves as a chloride ion pump, and sensory rhodopsin (SR), which is the receptor for phototaxis. The genetics, chemistry, conformation, photoreactions and regulation of these halobacterial proteins were explored at a recent workshop*. Discussion of the proteins was enriched by comparing them with rhodopsin, the photoreceptor protein in the rod cells of vertebrate retinas. Reports of a phototaxis receptor in a eucaryotic green alga² and the three cone pigments mediating human colour vision contributed to the liveliness of the meeting.

The lateral position of the retinal group of bacteriorhodopsin has been investigated by neutron diffraction of membranes containing hydrogenated or specifically deuterated retinal (N. A. Dencher, Berlin). Peaks in neutron difference maps fit nicely with the proposed position of the C-terminal helix, which contains lysine 216, the residue joined to retinal by a protonated Schiff base linkage (R. Henderson, MRC Cambridge). X-ray and electron diffraction patterns of M412, the deprotonated species in the proton-pumping photocycle, are very similar to those of protonated BR. The extent of structural change corresponds to a movement of one or two amino acid residues by a distance of about 4 Å. A rotation or tilting of an entire helix is excluded by these data.

The configurations of key bonds in the retinal group of bacteriorhodopsin attracted much interest. Resonance Raman spectroscopy and solid-state ¹³C-NMR have provided a wealth of information concerning the precise structural changes of the chromophore during the photocycle^{3,4}. Theoretical calculations indicate that rotation about the C14–C15 single bond is severely hindered in the protonated state. This single bond is thought to be essentially free in the unprotonated state, as well as in a protonated state, with a negative counter-ion near the Schiff base nitrogen (K. Schulten, Munich). Rotation about the C14–C15 bond may be an important gating mechanism in proton pumping by bacteriorhodopsin. Fourier transform infra-red spectroscopy has shown that several carboxylates undergo

changes in the photocycle (F. Siebert, Freiburg). It seems likely that the pathway of proton pumping will be known in atomic detail before long.

Halorhodopsin, discovered several years after bacteriorhodopsin⁵, pumps chloride ions into halobacteria⁶. Three different classes of chloride binding sites have recently been identified (B. Schobert, University of California, Irvine; T. Yoshizawa, Kyoto). Because it is highly specific for halide ions, site II is probably directly involved in transport. Moreover, occupancy of this site leads to a large spectral shift of the retinal chromophore. Site I affects the *pK* of the retinal Schiff base, whereas site III is less specific and has lower affinity for chloride. The photocycle of halorhodopsin resembles that of bacteriorhodopsin apart from the absence of a deprotonated intermediate. Instead, the most blue-shifted species is thought to arise from the interaction of the transported chloride ion with the protonated Schiff base nitrogen (D. Oesterhelt, MPI Martinsried). Halorhodopsin probably transports chloride into the cell by a mechanism very similar to that employed by bacteriorhodopsin to pump proton out of the cell.

Phototaxis

Halobacterium halobium is attracted to long-wavelength visible light (the red response) and repelled by short-wavelength light (the blue response)⁷. Halobacteria reverse their swimming direction when they encounter an increase in blue light intensity or a decrease in red light intensity. The isolation of a mutant devoid of BR and HR has led to the discovery of a third light-activated retinal protein in halobacteria⁸. This pigment has a photocycle akin to that of BR and HR, but it occurs on a slower time scale, and so was initially called slow-cycling rhodopsin (SR)⁹. Later studies of SR revealed it to mediate the attractant red response (PS565). Hence, SR now stands for sensory rhodopsin.

What about the receptor for the repellent blue response? The surprise is that SR does double duty, serving as both the attractant and repellent receptor (J. L. Spudis, Albert Einstein, College of Medicine¹⁰; R. A. Bogomolni, University of California, San Francisco). Absorption of a red photon by the dark-adapted state of SR (SR587) converts it into a form (SR373) that absorbs maximally at 373 nm and leads to an attractant signal. Absorption of a blue photon by SR373 then gives rise to a repellent signal. The repellent response is much more sensitive than the attractant response. Indeed, excitation of a single molecule of SR373 appears to be sufficient

to generate a reversal-inducing repellent signal. A key experiment leading to this proposed photochromic mechanism for the dual role of SR is that the repellent response to blue light is dependent on the presence of red background illumination. An alternative view, espoused by some aficionados of halobacteria phototaxis, is that different receptor proteins trigger the attractant and repellent responses¹¹. After a spirited evening discussion, it was agreed that a mutant lacking SR is needed to settle the issue. The effects of blue and red flashes separated by different intervals should also be highly informative. My feeling is that the photochromic mechanism is basically correct, and that halobacteria have exploited the convertibility of retinal isomers to get primitive colour-sensing from a single retinal protein.

Image-forming eyes are present in four phyla — molluscs, annelids, arthropods, and vertebrates. The action of photo-excited rhodopsin (R*) is best understood in vertebrates, where it triggers a cascade that leads to the hydrolysis of cyclic GMP (L. Stryer, Stanford University; M. Chabre, University of Grenoble). In the first stage, a single molecule of R* catalyzes the exchange of GTP for GDP bound to hundreds of molecules of transducin (T), a multisubunit peripheral membrane protein¹². GTP then activates a cyclic GMP phosphodiesterase. Hydrolysis of bound GTP brings transducin back to the dark state. This cycle is driven by the phosphoryl potential of GTP, which is nicely partitioned between the activation of transducin and the subsequent hydrolysis of its bound GTP. The restoration of the dark state also requires that R* be deactivated, which is accomplished by the phosphorylation of multiple serines and threonines in its carboxy-terminal domain (H. Kühn, KFA, Jülich). Phosphorylated rhodopsin is then capped by a 48K protein, which probably prevents it from interacting with transducin. Infra-red light-scattering studies of magnetically-oriented rod outer segments have shown that a molecule of R* activates a molecule of transducin in 1 msec, which is sufficiently rapid to enable transducin to participate in visual excitation¹³. The activation of transducin and the consequent hydrolysis of cyclic GMP are only part of the story. The challenge now is to delineate the links between this cascade and the closure of sodium channels in the plasma membrane. Calcium, is almost certainly involved, but its relationship to the cyclic GMP cascade is still an enigma.

The amino acid sequence of bovine rhodopsin and of the γ -subunit of transducin have been determined (Y. Ovchinnikov, Shemyakin Institute, Moscow). The sequences of bovine rhodopsin is not similar to that of bacteriorhodopsin, although there are similarities in their architecture¹⁴. Furthermore, vertebrate rhodopsin is like bacteriorhodopsin in the first few picoseconds of its photo-

*Molecular Biology of Retinal Proteins, Schloss Ringberg (near Munich), 10–14 September 1984.

chemistry. Thereafter, the role of photo-excited rhodopsin is to trigger an enzymatic cascade, whereas that of bacteriorhodopsin is to pump a proton and recycle rapidly. It will be interesting to see whether the rhodopsins of molluscs and arthropods are like those of vertebrates, as hinted at by the finding that squid rhodopsin can activate vertebrate transducin (H. Saibil, University of Oxford).

The genes for the three visual pigments mediating colour vision in humans have been isolated (J. Nathans, Stanford University) by a tour de force that began with the isolation of the gene for bovine rhodopsin¹⁵, which was then used as a hybridization probe to isolate the human rhodopsin gene¹⁶ and has now been similarly used to isolate the three genes for the colour pigments: two (probably the red and green absorbing pigments) are on the X-chromosome and the other (probably the blue absorbing pigment) is on an autosome.

Sequence analysis of the bovine and human rhodopsin genes had shown that they have the same intron-exon arrangement and that the two proteins have the same number and a 94 per cent match of amino acids. Moreover, the retinal attachment site, the two glycosylation sites, all but one of the phosphorylation sites and, most strikingly, the three loops on the cytoplasmic face of bovine rhodopsin are perfectly conserved; these loops almost certainly form the binding sites for transducin and other proteins that interact with photoexcited rhodopsin, such as rhodopsin kinase and the 48K protein. The intron-exon arrangements of the colour vision genes are like those of rhodopsin but the amino acid sequences have diverged considerably. Because the colour pigments have not yet been purified and sequenced the complete nucleotide sequences of their genes are eagerly awaited. Thomas Young, who in 1802 proposed that colour vision is mediated by three distinct receptors, would no doubt have derived enjoyment and satisfaction from knowing that their genes are now in hand. □

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Neurosciences

Spanish reflections and Cajal

from A. Claudio Cuello and Alan Cowey

IT IS no exaggeration to say that Don Santiago Ramon Y Cajal laid the foundations of modern neurobiology. Certainly he has been the most important scientist ever produced by Spain and his name still possesses some magical appeal, fifty years after his death. To mark that anniversary, the Cajal Institute of Madrid organized a meeting of neuroscientists in the elegant setting of the Consejo Superior de Investigaciones Cientificas, Madrid, from September 17–20 1984.

Neuronal connectivity, which could be described as having been discovered by Cajal, was given pride of place in a session that showed with extraordinary clarity how much modern techniques of labelled axoplasmic transport and immunocytochemistry, combined with the Golgi methods used by Cajal himself, are revealing about specific connexions and their development. Regeneration in the central nervous system, another of Cajal's abiding interests, was discussed in relation to new surgical techniques, to models of how appropriate connexions are formed, and to the exciting new evidence that intracerebral grafts may not only survive, but function. A session on synaptic transmission provided clear evidence of the extent to which synaptic events are now explained in terms of the molecular structure of receptors, and the development of nerve cells can now be attributed to electrical oscillation and resonance. Even Cajal could not have foreseen the final symposium on genetic engineering in neurobiology in which several examples were presented of how it is now possible to identify genes whose expression is limited to identified neurones, and how a given gene product can influence the physiology of a cell.

It was refreshing to see so many young Spaniards eagerly following these accounts of recent developments in the neurosciences, during which there were repeated references to the outstanding contributions made by 'Don Santiago' and his followers, a measure of the permanence of his school in contemporary neurobiology. So it is ironic that Spaniards now have to look abroad to enable themselves to keep pace with developments in this field. The exodus of eminent neuroscientists from Spain started early this century and was aggravated by the Civil War. Pio del Rio Hortega left for Oxford and then moved to Argentina where he generated great interest in neurohistology. Lorento di No emigrated to the United States, where he continued his timeless work on neuronal structure. Among countless other Spanish neuroscientists distributed among their adopted countries are J. Palacios in Swit-

erland, C. Sotelo in France, M. Marin-Padilla, A. DeBlas and M. Nieto Sampedro in the United States, and F. Cervero in Britain.

Will any of these distinguished scientists ever return to the homeland and contribute to the development of neurosciences in the land of Cajal? Probably not. The most realistic possibility for Spain to regain a proper international place in neurobiology would be for young neurobiologists to be offered good possibilities to carry out advanced research projects at home. A good number of Spanish scientists are currently trained abroad and some of Spain's more mature scientists are making tremendous efforts to expose themselves to new ideas and techniques. For all this to prosper there is a need for a firm intention to consolidate these gains, together with a sustained effort to finance the fresh demands these investigators will make. If the sometimes passionate words of the President of the Consejo Superior de Investigaciones Cientificas and the Ministers of Education and Health expressed on the occasion of Cajal's anniversary are taken literally, then we can expect a revival of the Cajal legend to have a positive impact on Spanish neuroscience. But a sincere intention will need to be behind the rhetoric. Cajal's gigantic figure should serve Spain as a source of continuous inspiration and not just simply a glorification of the past. □

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100 years ago

A METEOR VISIBLE IN THE DAYTIME

AT Waterpark, just below Waterford City, about 4 p.m. on October 15, my attention was attracted by a flash of increased light. Looking up, I saw in the south-south-west, about half way between the horizon and the zenith, a bright meteor slowly sailing nearly due west, its apparent size about half of the full moon, intensely white in colour at the centre, passing into blue at the circumference. It described a low arc, and was in sight for several seconds, leaving a trail of indigo blue with lighter luminous edges. The meteor disappeared behind some clouds which concealed the sun at a considerable altitude above the horizon.

From *Nature* **31**, 102, 4 December, 1884.

Seismology

First results from Japanese network for earthquake prediction

From Y. Okado.

A VERY extensive high-quality digital network for micro-earthquake and ground-tilt observation has recently been completed in the south Kanto-Tokai area surrounding Tokyo. The first scientific results are now appearing and allow, among other things, a remarkable three-dimensional picture of the earthquake hypocentre distribution to be produced. The distribution maps out the boundaries of the three plates that meet under this region of Japan^{1,2}.

The new network — perhaps the most extensive in the world — has been built primarily to aid the prediction of earthquakes in an area that contains a large fraction of Japan's industry and population. Construction began in 1978 aided by the passage of the Large Scale Earthquake Countermeasures Act which provides for the preparation of specific measures to be taken when an earthquake of magnitude 8 or more is predicted to be imminent. The network will also provide many data of great scientific interest because it is monitoring seismic activity in the unique triple junction of the Eurasian, Philippine and Pacific Plates. It is at the boundaries between these plates that great earthquakes have repeatedly occurred in the past. It is thought that an interplate

earthquake with a magnitude of 8 or more will occur in the Tokai region in the near future and a long-term prediction has already been issued to the general public.

The network, which has the National Research Centre for Disaster Prevention (NRCDP) in Tsukuba Science City at its hub, consists of 71 stations (Fig. 1) including three deep borehole observatories, 48 borehole stations approximately 100 m deep and 20 surface stations covering an area of approximately $350 \times 150 \text{ km}^2$ with an average distance of 25 km between adjacent stations. Among them, three deep borehole observatories which reach the basement rock at depths of 3,510 m, 2,330 m and 2,710 m, were completed in 1980 in and around Tokyo to overcome the artificial noises at the surface in the capital area. Most of the stations are equipped with a three-component set of velocity-type seismographs with a natural frequency of 1 Hz. A two-component borehole tiltmeter is sited at 23 stations, some of which are also equipped with special instruments such as three-component borehole strainmeters, acoustic emission sensors, ground-water-level meters and geochemical sensors.

All the observed data are digitized and transmitted with the pulse code modu-

lation method through telephone lines to NRCDP. The telemetry system uses various modern techniques such as automatic network timing (ANT) and wide dynamic range quantization (78 dB for 80 Hz sampling seismic data and 96 dB for 1 Hz sampling tilt data), and provides a density of transmission — 9,600 bits per second — that has never before been achieved by a conventional telemetry system. ANT uses a set of subclocks which generate echo-back signals to monitor continuously the turnaround times of each station and automatically adjust their midpoints to coincide with a master clock. By this method, the data sampling at all stations can be synchronized with an error of less than a few milliseconds and the problem of telemetry delay-time can be solved.

At the centre in Tsukuba, the outputs of all vertical seismograph and tilt data are continuously monitored on long-term chart recorders, while other components of the seismograph are recorded on different charts by the trigger method. Stress is put on quick and reliable data processing in order to allow short-term prediction. One computer system, directly connected with the telemetry system, carries out the filing

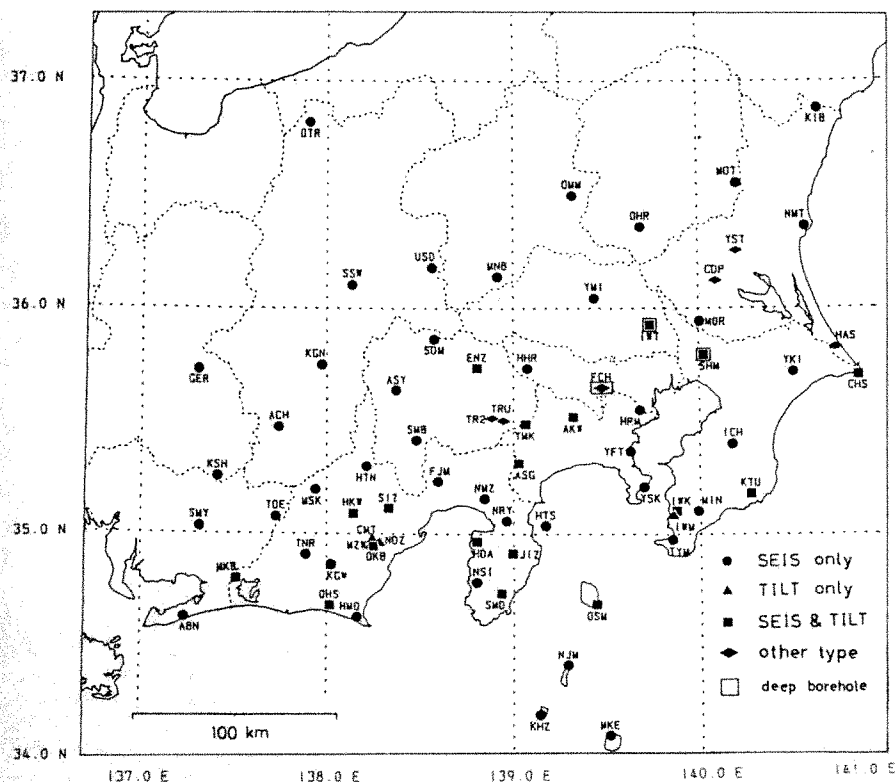


Fig. 1 National Research Centre for Disaster Prevention network for microearthquake and ground tilt observation (the southernmost station HCJ at latitude 33.1°N is omitted).

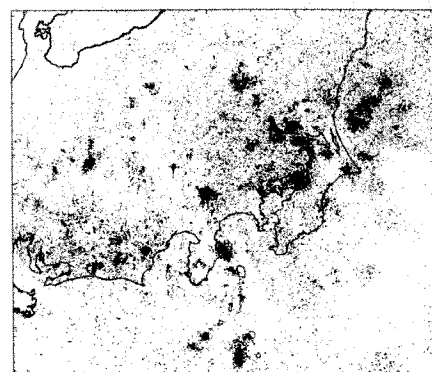


Fig. 2 Distribution of earthquakes in the Kanto-Tokai region located by NRCDP in the 5 years from 1 July 1979 to 30 June 1984. The plot shows about 28,000 earthquakes, most less than 3 in magnitude. (Latitude and longitude as on Fig. 1).

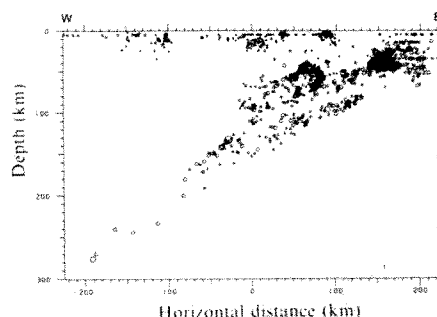


Fig. 3 East-west cross section of hypocentres of 1,515 earthquakes detected in the period 1 November 1982 to 31 October 1983 within 25 km on either side of the 36.25°N line. The Pacific plate is plunging underneath the Eurasian plate, while the Philippine Sea plate is being subducted from the south and collides with the Pacific plate at a depth of about 60 km making an 'earthquake nest' under the Kanto region.

of observed data and monitors the crustal activity on a real-time basis. Further data-processing steps, including hypocentre determination by rationalized semi-automatic methods, are executed by a second computer system.

Routine work for hypocentre determination began as soon as the first parts of the system were completed in July 1979. Every day around twenty earthquakes are located, most of them micro-earthquakes with magnitude around two. Figure 2 shows the hypocentres in the Kanto-Tokai area which have been detected by our network in the past 5 years and figure 3 shows the hypocentres in a vertical section through 36.25° N (ref.3). The vertical section clearly shows the Pacific Plate diving down underneath the Eurasian Plate upon which the main Japanese island

stands. The precise study of three-dimensional hypocentral distribution and focal mechanisms based on a large amount of accurate data leads us to an excellent image of the plate configuration in this complex region⁴. Also we have been able to measure a precursory tilt change in the 18 days preceding an earthquake that was of magnitude six and thirty one km away from the station⁵. □

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Earthquake prediction

Evaluating hydrological and geochemical anomalies

from Chi-Yu King

THE 27th International Geological Congress was held in Moscow from 4-14 August 1984 and, like many other recent geoscientific meetings, included earthquake prediction as a theme. Among the papers presented were five based on hydrologic and geochemical studies by Soviet scientists, providing some current views of the Soviet effort in this relatively new field of study. While some scientists consider the hydrochemical and geochemical methods highly effective for short-term prediction, others remain sceptical.

Substantial efforts to predict the time, location and magnitude of earthquakes with reasonable precision began about two decades ago in the Soviet Union, Japan, China and the United States. The Soviet studies were triggered by the destructive Khatay earthquake of 1949 in the Garm region of Tajikistan. Hydrochemical and geochemical methods began to draw attention when the 1966 Tashkent earthquake was found to have been preceded by anomalous radon changes in groundwater. Currently, a total of 240 wells are being monitored at 83 stations in practically all USSR seismic regions. About 40 parameters are being measured, including water level (or flow rate), temperature, electric conductivity, ion content, isotopic ratios (such as D/H, ¹⁸O/¹⁶O), and the presence of gases (such as Rn, He, H₂ and Hg vapour) either dissolved in groundwater or emanating from the ground. Many anomalies have been recorded before large earthquakes as far away as several hundred kilometres. The amplitude, duration and spatial extent of the anomalies are generally found to increase with earthquake magnitude and hence provide a means of prediction. Some earthquakes are even

claimed to have been predicted on the basis of hydrochemical geochemical and other data, but insufficient data were shown to permit a critical assessment of such claims.

Are the hydrochemical and geochemical changes produced by earthquake-related fluid movements in the earth — such as increased upward flow of deep-seated water and gas, flow of water from one aquifer to another through tectonically developed cracks in the barrier between them or movement of pore fluid squeezed out of surrounding rocks into well waters? It is conceivable. But these changes may also be the result of other environmental factors, such as rainfall, barometric pressure and groundwater pumping. Consequently, it is difficult to distinguish true earthquake-related anomalies from noise. Identification of anomalies is complicated further by the local geology of the monitoring sites, the lack of detailed understanding of fluid movement in the ground and data contamination by errors introduced in sampling and measurement. Most researchers in the Soviet Union and elsewhere agree that, to pursue earthquake prediction properly, it is necessary to use data recorded over a number of years with a variety of different instruments.

China initiated a massive earthquake-prediction program shortly after the 1966 Xingtai earthquake, which was the first of nine large earthquakes (magnitude ≥ 7.0) that devastated many areas on the mainland between 1966-76. The Chinese effort has mobilized tens of thousands of people, both professional and amateur, and has used many different methods, both modern and traditional. About 4,000 wells have been used for hydrochemical and geochemical monitoring, but only a few per

cent of them have been found useful. Because of the timeliness of the effort, a large number of scientific data and eyewitness accounts have been collected, many of which describe hydrochemical and geochemical changes. As in the Soviet Union, numerous pre-earthquake anomalies have been recorded at epicentral distances of up to several hundred kilometres in China and some of the data in combination with macroscopic phenomena and other kinds of data have been used successfully to predict several earthquakes including those in Haicheng in 1975 and Songpan in 1976. However, because of the aforementioned difficulties, it will not be possible to assess critically the relation of many of the reported anomalies to subsequent earthquakes until more data are published.

The Japanese earthquake-prediction program was implemented shortly after the destructive 1964 Niigata earthquake. Hydrochemical and geochemical observations began in 1973 and are carried out mostly in the Tokai region by the Geological Survey of Japan and several universities, with the help of some amateur groups. Anomalous changes have been reported in association with some earthquakes including that in Izu-Oshima-Kinkai in 1978.

In the United States, research on earthquake prediction has intensified since the Alaskan earthquake of 1964. A national program was implemented in 1977, with field effort concentrated in geophysical (especially, seismic and geodetic-strain) measurements along the San Andreas fault system in California. US hydrochemical and geochemical studies have been relatively few and have mostly involved simple inexpensive techniques and about 70 existing wells and springs, which may not always be suitable for earthquake prediction. There have been some moderate earthquakes in California in the past years, but few reliable hydrochemical, geochemical or geophysical anomalies were recorded. Some attribute this to the tectonic environment: the San Andreas fault system is characterized by simple shear movement with little stress concentration, whereas seismic regions in the Soviet Union and China are characterized by complex continental structure in a highly compressed state and earthquake-related anomalies may occur at the structurally singular points where stresses concentrate. Most of the earlier US results were published in the *Journal of Geophysical Research* in June 1980 and *Geophysical Research Letters* in May 1981.

Approximately 30 papers on earthquake hydrology and chemistry, including some presented at the 27th International Geological Congress, will be published in early 1985 in a special issue of *Pure and Applied Geophysics*. □

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Immunology

Exclusive immunoglobulin genes

from Frederick W. Alt

ON PAGE 517 of this issue, Ritchie, Brinster and Storb describe an elegant experiment¹ that provides strong support for one mechanism of the phenomenon known as allelic exclusion, which has traditionally been as puzzling an aspect of immunoglobulin genes as has their ability to specify a limitless diversity of antibodies.

Allelic exclusion, which is unique to immunoglobulin and related genes, means simply the expression of only one of the pair of genes that have the potential to specify a given chain of the immunoglobulin molecule^{2,3}. Although the function of allelic exclusion is still debated⁴, the simplest view is that the generation of mono-specific B-lymphocytes is an important pre-requisite to an immune system whose specificity is based on clonal selection, and that a mechanism has evolved to ensure such specificity.

From the molecular analysis of the immunoglobulin genes, it at first seemed that the explanation of both the generation of antibody diversity and allelic exclusion might lie in the fact that the variable (*V*) region of the antibody molecule is encoded in separate segments of DNA that are brought together in different combinations by rearrangement in the course of B-cell differentiation. This process accounts for much of the diversity of antibodies and, being error-prone, can be shown to result in the assembly of a non-functional gene. Thus it was conceivable that the probability of a functional rearrangement is low enough to make it very unlikely that two functional alleles would be generated. This explanation of the phenomenon of allelic exclusion is known as the stochastic model. There were, however, always problems with the simplest form of this model; Ritchie *et al.* now provide definitive evidence for an alternative model, known as the regulated model. By transfecting early mouse embryos with an already rearranged gene for the kappa (κ) light chain — one of the immunoglobulin chains — they have produced transgenic mice that express these genes in their B lymphocytes. To understand the implications of these new data, some background is necessary.

Each B lymphocyte displays on its surface an immunoglobulin molecule of a single specificity which is composed of a complex of two identical heavy chains and two identical light chains, the amino termini of which interact to generate the specific antigen binding pocket. The variable region of each light chain is encoded by a variable (*V*) gene segment and a joining (*J*) segment which are separate in the germ line DNA but are assembled somatically to form the complete variable region gene. There are two families of light-

chain genes, kappa and lambda, only one of which is expressed in any one cell. Immunoglobulin heavy-chain variable genes are also assembled from separate segments but in this case, there are three: the *V*, *D* (diversity), and *J* segments; the *D* to *J* complex is formed first and a *V* segment is added later to form the complete gene. (for review of *V* gene assembly see ref. 5). During B-cell differentiation, heavy chain gene assembly and expression generally precedes that of light-chain genes⁶.

Early speculation on the mechanism of allelic exclusion favoured a model in which only the 'allele' would be rearranged in a given B-cell clone, the other 'allele' remaining in germline configuration. But it quickly became apparent that within a population of normal immunoglobulin producing B-lymphocytes, significantly more than half of the κ light-chain alleles and nearly all of the endogenous heavy-chain alleles were rearranged at the *J* locus^{5,8}. Further analysis, however, revealed that one of the rearrangements was usually non-functional; for example, because of flexibility in the exact point of joining, the different segments sometimes end up in different translational reading frames. This led to so-called stochastic models for allelic exclusion, which postulated that because of the preponderance of non-functional rearrangements over functional rearrangements, the probability of achieving two functional rearrangements in one cell was very low.

Regulated model

Such models, however, do not easily explain either why a significant percentage of the 'second' alleles in κ -producing cells are in the germ line configuration or why allelic exclusion is almost always observed. Moreover, in κ light chain-producing cells with dual *VJ* rearrangements, both rearranged genes may be transcribed, and in several characterized cases both transcripts have been shown to be translated. Yet only one transcript in each case encodes a light chain that combines with the cellular heavy chain to produce the immunoglobulin molecule^{7,9}. This led to an alternative hypothesis; namely that allelic exclusion is effected by a 'signal' that is produced when rearrangement generates a light chain capable of combining with the pre-existing cellular heavy chain to form a functional immunoglobulin molecule. Such a signal would feed back and prevent further rearrangement. According to this model, if the first rearrangement were 'functional', a second rearrangement would be prevented — leading to a cell with one unrearranged κ allele. Were the first rearrangement to be 'nonfunctional', then a second could occur, — yielding a cell in which both alleles

are rearranged. Although this 'regulated' model seems to account for the various patterns of immunoglobulin gene rearrangement or expression that have been observed in tumour cells or normal cell populations, until now direct evidence has been lacking.

The transgenic mouse system provides an ideal experimental test of the predictions of such a model. In order to analyze the κ chains and the rearrangement of their genes, Storb and her colleagues made hybridomas from individual B lymphocytes obtained from the spleens of the transgenic mice. The regulated model predicts that expression of a transgenic κ chain in association with heavy chains in the B-cells (represented by individual hybridomas) of the transgenic mice, would prevent rearrangement of the endogenous κ genes. This is precisely consistent with the data from the hybridomas. Likewise, the regulated model predicts that if cells secrete an endogenous κ chain in the form of complete immunoglobulin, then the introduced gene will either not be expressed or will not associate with the endogenous heavy chain; several examples of the former phenomenon were observed. Most significantly, Ritchie *et al.* have not found any hybridoma that secretes the inserted κ -gene product in complete immunoglobulin and which has rearranged its endogenous κ genes. This is totally consistent with a regulated model of light-chain allelic exclusion.

A variety of evidence suggests that heavy-chain allelic exclusion is also mediated by a cessation of *V* assembly once a functional *VDJ* gene is assembled and expressed; among the evidence is the large percentage of frozen *DJ* intermediates at the 'non-functional' heavy-chain allele in populations of normal immunoglobulin-producing B-cells¹¹. Complete and active heavy chains have also been introduced into the germline of transgenic mice and shown to be expressed only in the appropriate lymphoid tissues¹²; in experiments designed to investigate the effect of the expression of the introduced gene on endogenous heavy chain gene rearrangement, pre-B lymphoid cell lines established from the bone marrow of the mice by transforming the cells with the Abelson murine leukaemia virus. Such cell lines, when generated from normal mice, almost always have dual rearrangements of both *J* alleles¹⁰. However, D. Weaver and D. Baltimore (personal communication) find that in cells from mice which express the heavy chain of the introduced gene, a substantial proportion of the heavy-chain alleles remains in the germline configuration. Although this striking finding is most easily interpreted as supporting a regulated model of heavy-chain allelic exclusion, other explanations — such as a difference in the populations of potential viral targets in the transgenic and normal mice — have not been excluded.

Thus the use of mice harbouring introduced genes has provided strong evidence for the concept that heavy-chain

and light-chain allelic exclusion is very strictly regulated and not simply a by-product of error-prone rearrangement. Although the existing evidence implicates the complete heavy chain and the complete immunoglobulin molecule, respectively, as the primary signals for heavy-chain and light-chain allelic exclusion, we are still far from understanding the molecular mechanism by which such signals are transmitted. Further insight may come from the observation that there are no consistent differences between the recombination/recognition sequences that flank the various heavy-chain and light-chain gene segments that are assembled at different points in B-cell ontogeny⁵. These findings suggest that all recombination events may be mediated by the same 'recombinase system'. If so, both allelic ex-

clusion and ordered rearrangement may be mediated by the accessibility of the substrate gene segments to a common recombinase rather than modulation of the activity of segment-specific recombinases. □

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Tumour development

Oncogenes in transgenic mice

from Douglas Hanahan

THE study of oncogenes is increasingly directed toward identifying the functions of their products and the molecular basis for the transformations they effect. One approach is to insert cloned (and sometimes deliberately altered) oncogenes into the germ line of an organism to establish their effect in that context. Two groups have recently illustrated how this approach can provide information on oncogene function and the development of cancers, whilst a third has demonstrated that a proto-oncogene can induce embryonal cells to differentiate.

In the October issue of *Cell*, Stewart, Pattengale and Leder¹ describe the occurrence of mammary tumours in transgenic mice which developed from one-cell embryos injected with hybrid oncogenes. The fusion genes comprised the long terminal repeat (LTR) sequence of mouse mammary tumour virus (MMTV) linked to various portions of the mouse *c-myc* proto-oncogene. The LTR carries transcriptional control sequences, including a transcriptional enhancer element and sequences which mediate induction of transcription by glucocorticoid hormones^{2,3}. The *c-myc* gene is the cellular homologue of the oncogene carried by MC29, an avian retrovirus², and is itself activated to participate in oncogenesis by retroviral insertion⁴ and by chromosomal translocation into the immunoglobulin locus^{5,6}. The *c-myc* gene product is a nuclear protein which binds DNA *in vitro*; its expression is apparently cell-cycle regulated, being strongly induced during the transition from the G₀ to the G₁ stage⁷.

Twelve of the thirteen transgenic mice obtained by Stewart *et al.* express the fusion gene in their salivary glands, several produce fusion gene transcripts in a few other tissues, and one shows constitutive

expression in all tissues. Each of the three females examined expressed the transgene in mammary tissue. The product of the fusion gene is the authentic mouse myc protein, which means it is difficult to distinguish from the product of the endogenous *c-myc* gene, but which allows the effects of quantitative differences in the normal protein to be examined.

Two of the original transgenic females developed spontaneous mammary adenocarcinomas while they were lactating. The tumours arose in one of the ten mammary glands of each mouse. There was no obvious DNA amplification or rearrangement in the tumour tissue. Fusion gene transcripts were abundant in the tumour relative to other tissues, as well as to transcripts from the endogenous *c-myc* gene. The development of mammary tumours was heritable from one of the two females, whose three transgenic female progeny developed similar mammary tumours during pregnancy. It is notable that the fusion gene produced tumours only in the tissue that is the primary target for MMTV. However, its penetrance is obviously incomplete, since the development of the cancer occurs only during pregnancy, does not arise in every mammary gland, and occurs in only a fraction of the independent strains harbouring the fusion gene. This implies that hormonal balance is in some way important and furthermore, that secondary events are necessary to elicit transformation. There were no discernable consequences of transgene expression on salivary glands of any of the twelve founder mice, nor on any other tissues found to be expressing the fusion gene.

In a somewhat analogous set of experiments, Brinster *et al.*⁸ have previously reported the transfer to the germ line of mice of the simian virus (SV) 40 T-antigen

'oncogene' (in the form of the early region of the viral genome in plasmids that also included either thymidine kinase or growth hormone genes^{9,10}, in both cases fused with, and so under the control of, the promoter of the mouse metallothionein gene). Transgenic mice were produced at frequencies comparable with those obtained with the fusion genes alone, which indicates that the presence of the SV40 T-antigen 'oncogene' is not necessarily deleterious to prenatal development. About 25 per cent of the transgenic mice develop tumours of the choroid plexus, a layer of epithelial cells which lines the brain and forms the blood-brain barrier. And virtually all of the original transgenic mice died prematurely. This phenotype is heritable, and is transmitted along with the T-antigen gene, which includes the normal SV40 transcriptional control regions.

The expression of the acquired transgene has several notable characteristics. The choroid plexus tumours, and cell lines derived from them, contain significant levels of SV40 T antigen and its corresponding message. However, no expression was detected in total brain tissue prior to tumorigenesis, although the presence of small quantities in the choroid plexus cannot be excluded. Furthermore, the metallothionein fusion genes are rarely expressed in most of these mice, which is in marked contrast to their frequent expression in transgenic mice that lack the accompanying SV40 early region^{9,10}.

Taken together these results suggest that the injected DNA (T-antigen gene plus metallothionein fusion gene) is inactivated early in development in some stable but reversible manner. There is a precedent for the stable inactivation and subsequent reactivation of the SV40 early region in cultured cells: a cell line carrying 25–50 copies of the intact SV40 early region, which produced no detectable T antigen following initial cloning and continued passage, subsequently expressed T antigen in an occasional cell, and when such cells were subcloned they were found to produce T antigen in a clonally stable manner¹¹. Several of the choroid plexus tumours and cell lines derived from them seem to contain amplified SV40 sequences, which suggests that amplification (and rearrangement) may be one mechanism for SV40 gene activation. The concepts of stable germ-line inactivation and subsequent somatic reactivation have been well documented in mouse strains harbouring genetically transmitted proviruses of murine leukaemia virus^{12,13}. In the case of transgenic mice, the inactivation affects not only the SV40 early region but also the normally active metallothionein fusion gene to which it is linked. The fact that only the choroid plexus is transformed may be accounted for by the tissue specificity of the SV40 transcriptional enhancer element.

In the studies of both Stewart *et al.* and Brinster *et al.*, the presence of an oncogene

in the germ line is not sufficient to produce a simple dominant phenotype. Rather, additional rare changes seem to be required for the oncogene to produce tumours. This has similarities to the evolving view of naturally-occurring cancers^{11,15}, where multiple events are most probably necessary to create the dominance that converts a normal cell into a tumour cell, and to explain the slow onset of transformation.

The production of transgenic mice harbouring new oncogenes requires that such genes do not perturb prenatal development. The work of Müller and Wagner¹⁶ nicely suggests that not all oncogenes do affect differentiation. They have transferred the *fos* proto-oncogene into F9 embryonal carcinoma cells, where it induces characteristic terminal differentiation.

F9 cells were originally established from a teratocarcinoma and have many qualities in common with cells of the early mouse embryo, as demonstrated by their ability to form aggregation chimaeras with normal embryo cells and their representation in somatic tissues of mice that develop from the chimaera. F9 cells can be induced by the combination of retinoic acid and dibutyryl cyclic AMP to undergo a series of alterations, resulting in epithelial-like cells that cease to divide and show many characteristics of authentic endoderm tissue¹⁷.

The *c-fos* gene, the cellular homologue of an oncogene (*v-fos*) found in several acute transforming viruses, produces a nuclear phosphoprotein¹⁸ that is expressed at very high levels in extra-embryonic tissues, especially amnion and visceral yolk sac¹⁹. When F9 cells are cotransfected with *c-fos* and an unlinked neomycin gene, which confers resistance to the drug G418, two classes of drug-resistant colonies arise. One consists of proliferating cells with normal F9 morphology. The other comprises enlarged, flat cells that assume an epithelial morphology and cease to proliferate. The flat cell colonies contain high levels of *c-fos* DNA, mRNA and protein.

The morphologically-altered cells develop an ordered array of intermediate filaments that carry several antigens characteristic of endoderm tissue. However, these cells do not produce either laminin or

α -fetoprotein, which are found associated with parietal and visceral endoderm, respectively. Fibronectin and type IV collagen are both detected in these cells — collagen is normally present in endoderm, while fibronectin is not. In contrast, retinoic acid induces the production of the intermediate filament network and type IV collagen, as well as either laminin or α -fetoprotein, but not fibronectin. Thus, the introduction and expression of the *c-fos* gene results in specific changes that are distinguishable

from those resulting from chemical induction. The ability to induce events characteristic of differentiation through the expression of a single transferred gene provides new opportunities for studying the role of gene expression (and its regulation) in some of the specific changes that are involved in complex this differentiation pathway. □

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Surface chemistry

Removing the black magic

from J.B. Pendry

MUCH of our chemical industry would not be economic without the presence of a catalyst, acting to short circuit energy barriers to the desired transmutation of one species of organic molecule into another and to discriminate against unwanted products. Frequently, the organic reactants are adsorbed on the surface of finely divided particles of a catalytic transition metal, the complex electronic structure of which offers a variety of possibilities for bonding, thus facilitating rearrangement of the reacting species. The key to understanding the catalyst's function lies in the configuration of the reactant molecules at the surface — that is in their crystallography. So thin and delicate is the monolayer of reactants that it is far from trivial to determine their crystallographic structure. It has only been in the last decade that surface crystallography can be said to have existed. At the International Conference on the Structure of Surfaces, 13-16 August, in Berkeley, California, Roland Koestner, Michel Van Hove and Gabor Somorjai of the university there, reported a major advance in crystallographic sophistication.

Apart from the detailed location of the hydrogen atoms, the Berkeley group was able to solve the complete crystallographic structure of hydrocarbon monolayers on platinum and rhodium surfaces formed by the adsorption of C_nH_{2n} , where n varies from 2 to 4. A great variety of structures form under different conditions. For example, it is possible to persuade one of the terminal carbon atoms to discard its hydrogen atoms in favour of bonding to the metal surface, settling into a three-fold hollow site available on the (111) single crystal surface used in the experiment. Some of the hydrogens are claimed by the unsaturated bonds further down the chain, forming the alkylidyne species $M_3C-(CH_2)_{n-1}-H$, which stands as close to vertical to the surface as the crooked C-C-C bond angle will allow (M_3 represents the three surface atoms to which the terminal carbon bonds).

For propylidyne and butylidyne species, a further wrinkle to the structure was

untangled. The crooked bond angles mean that $C-CH_2-CH_3$ adopts a configuration in which the $C-CH_2$ bond is perpendicular to the surface, whilst the CH_2-CH_3 bond points in some direction away from the surface. At low coverages and high temperatures this arm swings around in a random manner, but at higher coverages and lower temperatures free rotation is prevented by interaction with neighbours and an ordered structure results with the arms neatly folded into an ordered structure.

How was the Berkeley group able to make such precise statements about the surface crystallography? As with most surface experiments, several techniques were employed to cross check conclusions, but the most important was low energy electron diffraction. Electrons with energies around 150 eV have a wavelength of around 1 Å, like X rays. Unlike X rays, they interact strongly with matter giving good surface sensitivity. To obtain structural information from their diffraction patterns, a sophisticated theoretical interpretation is necessitated but the Berkeley group has achieved a level of sophistication that had been thought impossible. For butylidyne on platinum, with a $(2\sqrt{3} \times 2\sqrt{3}) R30^\circ$ unit surface cell, the coordinates of about 50 atoms had to be correctly included to an accuracy of about 0.1 Å.

Just as the journey from the original application of X rays to the structure of DNA was a long and difficult one, so there is a great contrast between the early determinations of coordinates for single atoms at surfaces, and the present work, although even butylidyne on platinum falls short of the complexity of DNA. New possibilities for studying complex systems are opened. In particular we are brought closer to an atomistic understanding of how reactions proceed at surfaces, which help to make the evolution of new higher yielding catalysts more a matter of the science of design than the black magic of trial and error. □

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Origin of the Solar System dust bands discovered by IRAS

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Measurements of longitudinal variations in the brightness and the latitude of the Solar System dust bands observed by the Infrared Astronomical Satellite determine the orbital elements of the particles involved. They may also allow discrimination between cometary and asteroidal models of the origin of these bands and even perhaps the zodiacal dust complex.

ZODIACAL light is observed as a faint glow in the west after sunset and in the east before sunrise. Conventionally, the debris of short-period comets is thought to give rise to the zodiacal cloud, the grains of which are responsible for zodiacal light. The sizes of particles emitted by comets^{1,2} are similar to those thought to scatter the zodiacal light^{3,4}, while the association of meteor streams with known comets^{5,6} demonstrates that comets inject some grains of diameter $\geq 1 \mu\text{m}$ into the interplanetary complex. In addition, the orbital elements of short-period comets generally^{3,7-9}, but not completely¹⁰, match what is known about the trajectories of interplanetary dust. A large fraction of interplanetary dust grains have low densities and airy structures similar to those expected for volatile-rich cometary particles which form in a low-gravity environment^{7,11,12}.

This hypothesis is, however, inconsistent with data showing that short-period comets are an inadequate source of debris^{10,13,14}, delivering only a few per cent of the mass needed to replenish that lost from the zodiacal complex through self-collisions and by orbital collapse due to Poynting-Robertson drag. To avoid this conflict, it has been proposed that asteroids^{10,15} rather than comets primarily supply the cloud. Alternatively, the zodiacal cloud may be episodic, being supplied intermittently by the capture of an abnormally large comet into a short-period orbit^{10,13}. A further explanation is that the current cometary mass input has been seriously underestimated because comets eject most mass in the size range of pebbles or boulders, which are difficult to detect. Infrared Astronomical Satellite (IRAS)^{16,17} and radar observations¹⁸ of macroscopic cometary debris support this possibility. IRAS observations of the zodiacal cloud may help decide between the above explanations. In particular, we demonstrate here that the Solar System dust bands, which IRAS showed to be superposed on top of the much stronger zodiacal signal, may be due to asteroid families; asteroidal dust could thus be a significant contributor to the remainder of the zodiacal signal. Unfortunately, the IRAS dust bands can also be interpreted as indicative of episodic cometary injection, where the latitudes of the bands would correspond to the inclination of the comet at the time of injection.

We show here that distinctive longitudinal variations in thermal flux and mean latitude can be used to determine the typical orbits of the grains comprising the Solar System bands. In particular, we predict how the bands should vary if they are debris associated with the three principal asteroid families. Based on these ideas, IRAS observations (after substantially more data analysis) may allow discrimination between asteroidal and cometary origins of the dust bands and, perhaps, of the entire zodiacal cloud.

Dust bands and asteroid families

IRAS discovered three comparatively narrow bands of far-infrared emission superposed on the spatially broad zodiacal background^{19,20}. The most prominent band lies in or near the ecliptic and is flanked by two other bands $\sim 10^\circ$ above and below it (see Fig. 1). These bands of dust have colour temperatures of 165–200 K. The derived distances of 2–3 AU suggest that they are approximately at the distance of the main asteroid belt and may be the results of collisions between asteroids¹⁹.

Hirayama's asteroid families²¹, defined on the basis of clustering in orbital parameter space (semimajor axis a , proper eccentricity e' and proper inclination I'), are also considered to be collision products. We show first that the latitude distributions of the asteroids in the three most prominent Hirayama families (Koronis, Eos and Themis) match the structure of the IRAS Solar System dust bands. The distribution of the main belt asteroids in the $a - \sin I'$ plane is shown in Fig. 2. The Koronis, Eos and Themis families are prominent in this plane and are equally well-clustered in the $a - e'$ plane (see Table 1). There are no other families, except perhaps the multiple Flora families at 2.2 AU, that are both as well-populated and as tightly bunched in orbital parameter space as these. The ages of the families are unknown, but because the longitudes of the proper nodes, Ω' , are randomly distributed and there are no correlations between Ω' and the nodal rates, $\dot{\Omega}'$, their ages are certainly $> T = 2\pi/\delta\Omega' \approx 10^6$ yr, where $\delta\Omega'$ is the observed dispersion in Ω' (see Table 1). If the families are the products of chance collisions and if asteroidal orbits are stable over the lifetime of the Solar System, then the presence of only three or four prominent families indicates that their ages are $\sim 10^9$ yr. We can make no definitive statements about the long-term stability of Solar System orbits: planetary perturbations may disperse the orbital elements of family members in a time short compared with the age of the Solar System, in which case the families could be quite young. This would also be the case if comminution of intermediate-size asteroids were important.

The distribution in latitude (integrated over all longitudes) of particles associated with the prominent families shown in Fig. 3, was obtained by distributing 50 test particles uniformly in time (or mean anomaly) on the orbits of each of the N members of each family. The central double spike in the composite distribution is due to the low-inclination families, Koronis and Themis, and has a total width (FWHM) of $\sim 4^\circ$. The wings of the distribution at $\pm 10^\circ$ are due to the Eos family, and arise because asteroids in inclined orbits spend a disproportionate amount of time at the extremes of their vertical

Table 1 Proper elements of Hirayama families

	N	a (AU)	e'	I' (deg)	$\dot{\Omega}'$ (arc s yr ⁻¹)	T (yr)
Koronis	42	2.875 ± 0.018	0.049 ± 0.006	2.12 ± 0.08	-66.7 ± 1.4	9×10^5
Eos	74	3.015 ± 0.006	0.071 ± 0.008	10.20 ± 0.28	-73.2 ± 0.6	2×10^6
Themis	62	3.136 ± 0.025	0.152 ± 0.009	1.42 ± 0.22	-104.4 ± 3.7	4×10^5

harmonic oscillations. Hence, a set of orbits with a common inclination and randomly distributed nodes will give rise to two apparent bands of particles symmetrically disposed above and below the mean plane of the system²⁰. There is some evidence that the central spike in Fig. 1 is a double spike; confirmation of this would demonstrate that this feature is associated with particle orbits with non-zero mean inclination and would rule out the alternative possibility that it is associated with dust which has settled to the invariable plane of the Solar System.

Size-frequency distributions

If the IRAS dust bands are located at ~ 3 AU, then from their optical depth $\sim 10^{-8}$ (ref. 19), we calculate that their total apparent area is $\sim 2 \times 10^{19} \text{ cm}^2$. The present population of small asteroids is probably the product of catastrophic collisions of larger asteroids²²⁻²⁴. The size-frequency distribution of these collision products can be described by a cumulative power law of the form

$$N(r) = \frac{1}{3(q-1)} \left(\frac{r_0}{r} \right)^{3(q-1)} \quad (1)$$

where $N(r)$ is the number of asteroids with radii $> r$ and r_0 is a constant. The theoretical equilibrium solution^{25,26} has a population index $q = 1.837$, a value which is supported by the observed distributions of small asteroids in both the McDonald and the Palomar-Leiden surveys²⁵⁻²⁷.

Since $5/3 < q < 2$, the total area, A , is dominated by contributions from the smallest particles of radii $\sim r_{\min}$, where $r_{\min}$ is the lower cutoff of the size distribution, whereas the total volume, V , is dominated by contributions from the larger asteroids. If we write $V = (4/3)\pi R_e^3$, then

$$A = \frac{\pi r_{\min}^2}{(3q-5)} \left(\frac{r_0}{r_{\min}} \right)^{3(q-1)} \quad (2)$$

and

$$r_0 = R_e [3(2-q)]^{1/3} \left(\frac{r_0}{r_{\max}} \right)^{2-q} \quad (3)$$

where $r_{\max}$ is the radius of the largest asteroid in the population.

The albedos of $\sim 40\%$ of the asteroids in the prominent Hirayama families are known²⁸, and we have used them to determine the mean albedo peculiar to each family. By assigning this mean value to those asteroids without measured albedos, we have calculated the total volume of each family (and hence R_e) from the absolute magnitudes²⁹ of the asteroids in that family. The values of r_0 calculated from the observed volumes are shown in Table 2, and the resulting power-law distributions [equation (1)] are compared with the observed cumulative distributions in Fig. 4.

The size distribution of asteroidal particles cannot be extrapolated to arbitrarily small sizes, as very small particles are destroyed by fast-moving particles associated with the cometary flux¹⁵. Dohnanyi¹⁵ estimates that asteroidal particles dominate the population of small particles only for $r > 1$ mm. If $r_{\min} = 1$ mm, then we calculate that the total area A of the prominent Hirayama families is $\sim 10^{19} \text{ cm}^2$ (Table 2). This is in satisfactory agreement with the estimated area of the dust bands. As we argue that the prominent asteroid families may provide the emitting area observed by IRAS, then the area of the entire asteroid belt might be expected to dominate the IRAS zodiacal signal. Our calculation of cross-sectional area, however, may not be applicable to the entire asteroid belt. Because the large asteroids are probably gravitationally-bound "rubble-piles"²⁴, they do not contribute to the population of small collision products. Accordingly, although the total volume of the prominent Hirayama families is only a few per cent of the total volume of the asteroid belt, the debris derived from the original catastrophic breakup of the parent asteroids or from the gradual

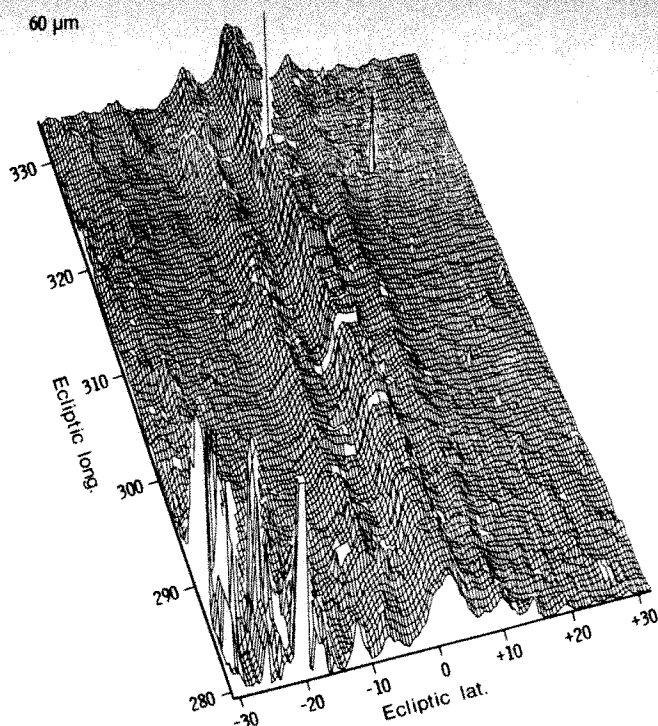


Fig. 1 IRAS data at a wavelength of $60 \mu\text{m}$ taken from scans across the ecliptic plane. The data are spatially averaged over 0.5° and the much stronger but smoother zodiacal contribution has been removed. Three major dust bands are found in, and on either side of, the ecliptic. From ref. 20.

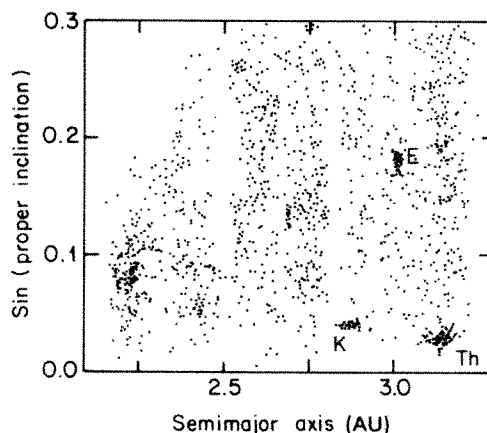


Fig. 2 The sine of the proper inclination and the semimajor axis of all asteroids listed in the TRIAD file, after ref. 34. The three most prominent families, Koronis (K), Eos (E) and Themis (Th) are identified.

Table 2 Size distributions of Hirayama asteroid families

Family	Albedo	R_e (km)	$r_{\max}$ (km)	r_0 (km)	A^* (cm^2)	$A^\dagger$ (cm^2)
Koronis	0.17 ± 0.05	43	23	37	2.0×10^{18}	6×10^{17}
Eos	0.09 ± 0.04	90	53	75	1.2×10^{19}	4×10^{18}
Themis	0.07 ± 0.03	124	99	98	2.3×10^{19}	7×10^{18}
Total					4×10^{19}	1×10^{19}

* $r_{\min} = 100 \mu\text{m}$.

† $r_{\min} = 1 \text{ mm}$.

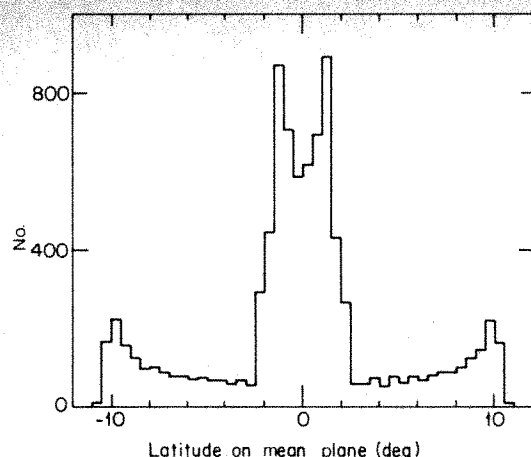


Fig. 3 The latitudinal distribution (integrated over all longitudes) of test particles spread uniformly in mean anomaly (or time) on the orbits of the known family members. 50 test particles were distributed along the orbits of each of the 42 Koronis family members, the 62 Themis family members, and the 74 Eos family members. The sum of the three distributions is shown. The latitudes are measured with respect to the mean plane of the Eos family, the orientation of which with respect to the ecliptic is determined by I_f and Ω_f (see Table 3). Because the three families have similar semimajor axes, the orientations of their mean planes are approximately the same.

comminution of the resultant family members may be much more prominent in the smaller size ranges that dominate the total cross-sectional area of the belt.

We consider that the latitudinal distribution of the prominent Hirayama families and their estimated total area make these asteroidal collision products the most likely candidate for the source of the Solar System dust bands observed by IRAS. We now describe further measurements that could be made to test this hypothesis.

Forced eccentricities and inclinations

Perturbations by Jupiter and, to a lesser extent, Saturn and the other planets result in periodic variations in the eccentricities and the inclinations of asteroidal orbits. The eccentricity vector of each orbit can be described as the vectorial sum of a free component of constant magnitude e' (the proper eccentricity) and a forced component of constant magnitude e_f , as follows:

$$e \cos \tilde{\omega} = e' \cos (\tilde{\omega}'_0 + \tilde{\omega}' t) + e_f \cos \tilde{\omega}_f \quad (4)$$

$$e \sin \tilde{\omega} = e' \sin (\tilde{\omega}'_0 + \tilde{\omega}' t) + e_f \sin \tilde{\omega}_f \quad (5)$$

Here, $\tilde{\omega}$ is the orbit's longitude of perihelion, $\tilde{\omega}'$ is the secular apsidal precession rate due to planetary perturbations and $\tilde{\omega}_f$ is the (slowly varying) forced longitude of perihelion. The constant $\tilde{\omega}'_0$ reflects initial conditions. The inclination vector can be described analogously in terms of a proper inclination I' , and a forced inclination and nodal longitude, I_f and Ω_f . To a good approximation, the secular nodal precession rate $\Omega' \approx -\tilde{\omega}'$. Table 3 lists the values of e_f , ω_f , I_f , and Ω_f for the three asteroid families discussed above. These quantities were calculated using the theory of Brouwer and van Woerkom^{30,31} including the effects of all the planets except Pluto, but in which terms smaller than e^2 and I^2 in the disturbing function are ignored. The errors produced by this truncation are less than 5% (J. G. Williams, personal communication). Greater accuracy could be achieved by using the higher-order of Williams³². In the low-order theory, the forced eccentricities and inclinations are independent of the free eccentricities and inclinations (assuming that these quantities are small) and depend only on the semimajor axis, a , of the orbit. Hence, measurements of the distortions of the

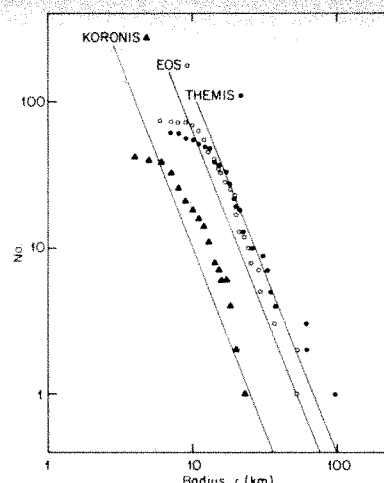


Fig. 4 Size-frequency distributions of the three prominent Hirayama families. N is the cumulative number of asteroids with radii $> r$. The intercepts of the theoretical straight-line fits are derived from the total volumes of the asteroids in each family. The slopes are $-3(q-1)$, where $q = 1.837$.

dust bands associated with the forced orbital elements may enable the distances of the bands to be deduced.

Geometrically, the forced inclination implies that all orbits with a particular value of semimajor axis precess about a common mean plane, whose inclination and ascending node are given by I_f and Ω_f , respectively. (This mean plane is close to, but not coincident with, the Solar System's invariable plane.) The peaks in the latitudinal distribution of an asteroid family (see Fig. 3) are symmetrically disposed with respect to this mean plane and, in a Sun-centred coordinate system, form two parallel bands around the sky separated by $2I'$. Variations in the apparent latitudinal separation of the bands as seen from the Earth,

Table 3 Orientation of forced eccentricities and inclinations with respect to the ecliptic

Family	e_f	$\tilde{\omega}_f$ (deg)	I_f (deg)	Ω_f (deg)
Koronis	0.0371	6.2	1.16	96.1
Eos	0.0373	7.6	1.19	97.1
Themis	0.0379	8.7	1.22	97.8

corresponding to changes in telescope pointing, also give a method of determining the distances of the bands³³. In this method, however, allowance must be made for the variation associated with the forced eccentricity, discussed below.

The orientation of the mean plane with respect to some reference plane (the forced inclination, I_f) and the longitude of the ascending node (Ω_f) are diagnostic of the semimajor axis a . Measured with respect to the Earth ecliptic and the equinox of 1950.0 (for observational purposes, a more convenient reference than the invariable plane), I_f increases from a minimum of 0.60° at 2.185 AU to 1.23° at 3.2 AU, while Ω_f increases from 315.2° at 2.0 AU to 98.0° at 3.2 AU (ref. 30). The appearance of the putative Themis band in a Sun-centred ecliptic coordinate system is shown in Fig. 5A. The sinusoidal variation of mean latitude with longitude has an amplitude of I_f and the phase of the variation is determined by Ω_f .

The effects of a forced eccentricity on the spatial distribution of an asteroid family or a dust band are analogous to those produced by a forced inclination and are illustrated for the

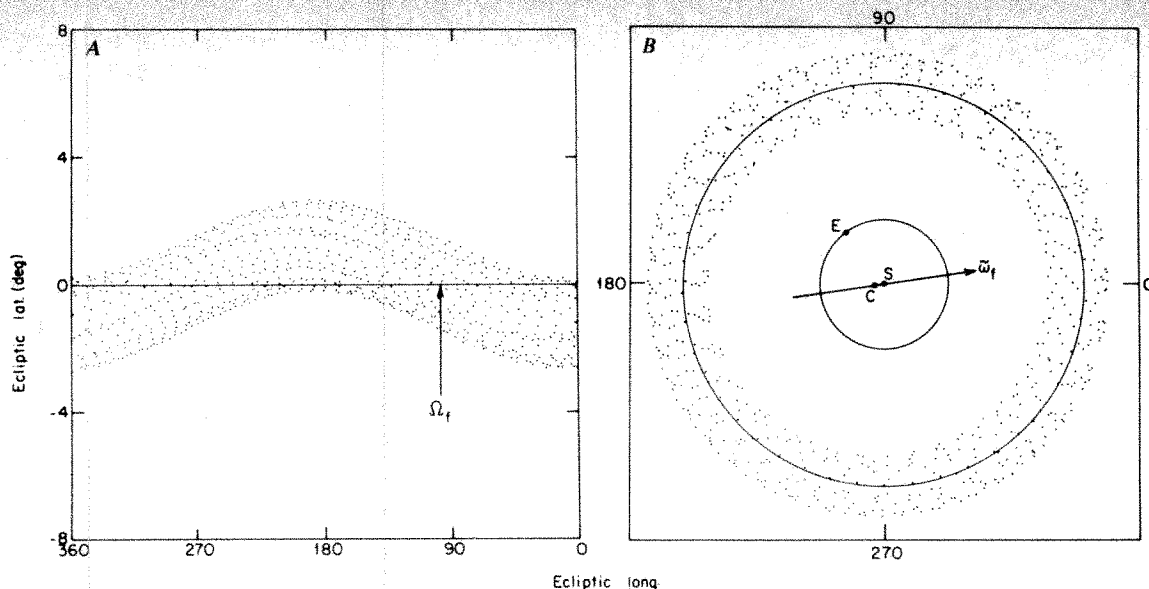


Fig. 5 Two views of the putative Themis dust band. The latitudinal width of the band in *A*, in a Sun-centred coordinate system, is $21'$ at all longitudes. The inner and outer envelopes of the particle orbits in *B* are circularly symmetric about a point *C* which is displaced from the Sun *S*, by a distance ae_f , in a direction opposite to that of the longitude of perihelion of the forced eccentricity, $\tilde{\omega}_f$. The radial width of the circular dust band is $2ae'$. The solid circle within the dust band is centred on *S* and has a radius equal to the mean semimajor axis, *A*, of the Themis family. The inner solid circle denotes the orbit of the Earth *E*. The striations in *A* are an artefact of the few points used in the plot and disappear as the number of points increases.

Themis band in Fig. 5*B*. The band is elliptical with an eccentricity e_f and a longitude of pericentre $\tilde{\omega}_f$; its radial width is $2ae'$. To first order in e_f , the inner and outer envelopes of the band are circles centred on a point *C*, displaced from the Sun by an amount ae_f in a direction opposite to that of $\tilde{\omega}_f$. The angular number density of the dust particles varies as

$$1 - 2e_f \cos(\lambda - \tilde{\omega}_f) \quad (6)$$

where λ is the ecliptic longitude, whereas the mean distance of the band from the Sun varies as

$$1 - e_f \cos(\lambda - \tilde{\omega}_f) \quad (7)$$

Thus, the number density of particles is a minimum in the direction of $\tilde{\omega}_f$, but at $\lambda = \tilde{\omega}_f$ the particles are both closer to the Earth's orbit and hotter. These effects more than compensate for the lower particle number density, so that the dust band is predicted to be brightest at pericentre.

Figure 6 shows the expected variation with ecliptic longitude of the thermal flux at the four wavelengths that correspond to the IRAS windows. This particular calculation applies to the Themis band, but calculations for Eos and Koronis give almost identical results. For this heuristic model, we have assumed (1) that the dust particles have an emissivity of 0.9, a geometric albedo of 0.1 and a phase integral of 0.6; (2) that the Earth's orbit is circular; and (3) that the pointing direction of the IRAS telescope is always tangential to the Earth's orbit. The mean temperature of dust particles associated with the Themis family is 160 K. At this temperature, the Planck function peaks at a wavelength of $\lambda = 18 \mu\text{m}$. At shorter wavelengths, the modest temperature change between aphelion and perihelion (~ 25 K) produces a comparatively large change in thermal flux and thus the longitudinal variation in flux is predicted to be strongest in the $12\text{-}\mu\text{m}$ window.

The phase of the longitudinal variation in brightness is determined by $\tilde{\omega}_f$ and is, therefore, diagnostic of *a*. The variations of e_f and $\tilde{\omega}_f$ with *a* are complex due to the existence of secular resonances at 2.03 AU (the S_6 resonance) and at 2.64 AU (the S_{10} resonance)³⁰. Ignoring the very large changes that occur close to these resonant locations, however, we conclude that between 2.2 and 3.2 AU, e_f generally decreases from ~ 0.054 to ~ 0.035 and then gradually increases to ~ 0.038 , whereas $\tilde{\omega}_f$

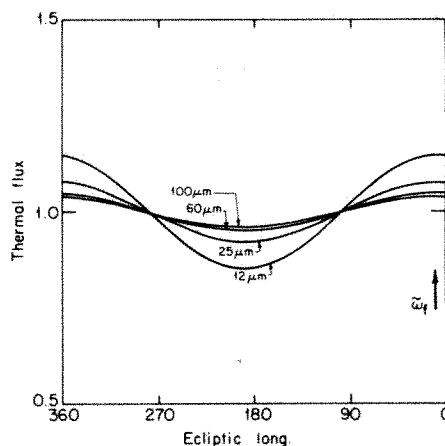


Fig. 6 Expected variation with Sun-centred ecliptic longitude of the thermal radiation from dust particles associated with the Themis family at wavelengths of 12, 25, 60 and $100 \mu\text{m}$. The arrow marks the longitude of perihelion of the forced eccentricity, $\tilde{\omega}_f$. The longitude refers to the heliocentric longitude of the dust particles, rather than to the observed geocentric longitude, although the flux is that observed at the Earth.

increases from 333.8° to 9.2° . The amplitude of the brightness variation is largely determined by e_f and is thus also diagnostic of *a*. The radial width of the dust band is determined by e' , and will affect both the apparent, that is, the flux-weighted, mean radius of the band and the mean colour temperature derived from brightness measurements in the four IRAS wavelength channels.

Conclusions

A detailed analysis of the IRAS data should yield information on all the orbital elements of the dust particles and hence should disclose the origin of the dust bands. We have shown here that the orientation of the mean plane of a band (or pair of bands) is determined by the forced inclination and nodal longitude of the particles' orbits, whereas the forced eccentricity and peri-

centre longitude lead to predictable longitudinal brightness variations. Each of these four quantities depends in turn on the semimajor axis, as long as e and I remain small. The angular separation of a pair of bands, as viewed from the Sun, provides a direct measurement of the proper inclination, I' . The remaining orbital element of interest, the proper eccentricity, e' , may be derivable from a careful study of the apparent mean radius and/or colour temperature of a band. These results are applicable to, and provide a means of testing, any specific dynamical model of the dust bands. Apart from the asteroid family model

Received 16 August; accepted 3 October 1984.

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presented here, the most plausible alternative model would involve the disintegration of two or more comets. In this case, however, the small- e approximations would require some revision; nevertheless, our approach should be equally suitable.

This research was supported by grants from NASA and NSF. The Infrared Astronomical Satellite (IRAS) was developed and operated by the Netherlands Agency for Aerospace Programs (NIVR), the United States National Aeronautics and Space Administration (NASA), and the United Kingdom Science and Engineering Research Council (SERC).

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Mechanism of colour discrimination by a bacterial sensory rhodopsin

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A photosensitive protein resembling the visual pigments of invertebrates enables phototactic archaeobacteria to distinguish colour. This protein exists in two spectrally-distinct forms, one of which is a transient photoproduct of the other and each of which undergoes photochemical reactions controlling the cell's swimming behaviour. Activation of a single pigment molecule in the cell is sufficient to signal the flagellar motor. This signal-transduction mechanism makes evident a colour-sensing capability inherent in the retinal/protein chromophore.

MANY bacterial species alter their swimming behaviour in response to environmental stimuli. Chemotactic bacteria, for example, sense particular nutritive and noxious substances and respond to them as attractants and repellents, respectively¹. *Halobacterium halobium*, a flagellated archaeobacterium which grows in saturated brine, exhibits chemotaxis and is also phototactic; the cells are attracted to long wavelength visible light (the 'red' light response) and repelled by shorter wavelength light (the 'blue' light response)². The first quantitative studies of these responses² revealed both sensitivities present simultaneously. In the absence of photostimuli, the cells move in a three-dimensional random walk, swimming short stretches in an approximately straight line and periodically reversing direction through roughly 180°. Light intensity gradients bias the random walk by modulating the frequency of reversals³. Increases in blue or decreases in red light intensity are interpreted by the cell as movement in an unfavourable direction causing reversal of swimming direction. The opposite intensity changes suppress reversals. As in the case of bacterial chemotaxis, the flagellar motor responses are transient; the cells adapt to the new light intensity and resume their prestimulus reversal frequency³.

Vitamin A aldehyde (retinal) forms the chromophore of visual

pigments and is required also for photosensory reception in halobacterial phototaxis^{4,5}. Two retinal-containing pigments, bacteriorhodopsin and halorhodopsin, function as light-driven ion transporters in *H. halobium*⁶. These pigments may be attractant receptors and their short wavelength photointermediates repellent receptors⁷. Mutants lacking both bacteriorhodopsin and halorhodopsin exhibit normal colour-discriminating phototaxis⁸, indicating that at least one additional retinal pigment must exist in this organism.

We have detected this third photoreactive retinal-containing membrane protein in *H. halobium*⁹. Like bacteriorhodopsin and halorhodopsin, photoexcitation of the third pigment is followed by a sequence of thermal steps returning the molecule to its original state. This cyclic process (or photocycle) has a half-time of ~0.8 s at 23 °C, in contrast to the faster (~10 ms) photocycles of bacteriorhodopsin and halorhodopsin. The slow-cycling rhodopsin does not appear to mediate active ion transport⁹ and is the only spectroscopically detected retinal pigment in phototactic strains which lack bacteriorhodopsin and halorhodopsin^{9,10}. Addition of retinal to a mutant blocked in retinal synthesis generates both slow-cycling rhodopsin and phototaxis with similar kinetics and retinal concentration dependence^{10,11}. These properties and its absorbance in the region of attractant

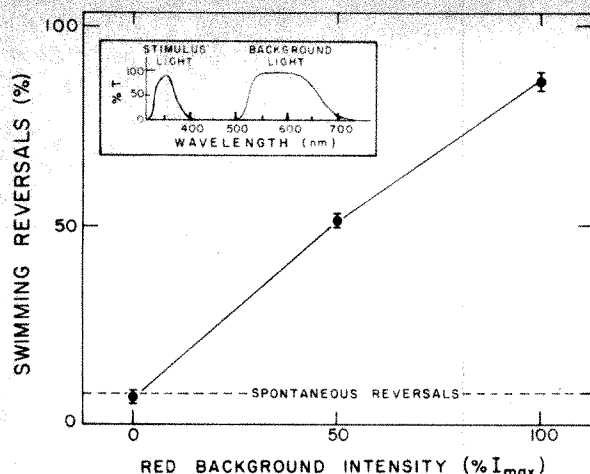


Fig. 1 Dependence of near ultraviolet repellent response on visible background light. The figure shows the % of cells reversing within 2 s of the near UV stimulus as a function of red background intensity. Points are average values $\pm$ extreme values of 2 separate determinations, each based on >40 cells. The inset shows the transmittance spectra of the filter combinations used for stimulus and visible background irradiation. The stimulus and observation light from a 150 W mercury (Hg) lamp and 50 W tungsten Zeiss microscope illuminator, respectively, were combined through a glass beam splitter which allowed passage of 30% of the infrared observation light (Kodak 87A filter) and 30% of the Hg lamp radiation. The combined beam illuminated the cells through the microscope phase condenser. The red background light was provided by an optically collimated 50 W tungsten light source reflected by a quartz beam-splitter mounted below the condenser. Light fluxes at the sample were measured with a Kettering 65 Radiometer and intensities calculated using irradiation areas measured with a calibrated ocular Zeiss micrometer. Light intensity was varied with Kodak neutral density filters. Stimulus light intensity, $1,400 \text{ erg cm}^{-2} \text{ s}^{-1}$. Maximum red background intensity (I_{max}), $7.2 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$. Infrared radiation, $>750 \text{ nm}$.

(red) sensitivity¹⁰ make this protein a likely candidate for the attractant photoreceptor. The repellent photoreceptor, however, poses a problem because no separate pigment active in the region of repellent (blue) sensitivity is detectable by sensitive flash photolysis analysis (detection limit 50 molecules per cell, unpublished data). The work we report here shows that a solution to this dilemma can be found within the slow-cycling rhodopsin photoreactions. The repellent receptor is expected to absorb maximally near 370 nm according to phototaxis action spectra^{2,4}; photoexcitation generates an intermediate species with maximal absorbance at 373 nm (S_{373}) within a few microseconds⁹ which, in addition to its slow thermal relaxation, can more rapidly reconvert to the red-absorbing form of the molecule (sR_{587}) by absorption of a second photon⁹.

We considered that slow-cycling rhodopsin in its S_{373} form might mediate the repellent responses. If this is correct, no repellent responses should occur in the absence of S_{373} and repellent sensitivity should appear when S_{373} is generated by illumination of sR_{587} . The work presented here shows these predictions are borne out and reveal a mechanism for colour-sensing based on the dual role of slow-cycling rhodopsin as attractant and repellent photosensory receptor.

Swimming behaviour

The absorption spectrum of sR_{587} is so broad that any visible light will generate S_{373} . Thus all published measurements of repellent responses have been made in the presence of S_{373} generated by the light used to observe the bacteria. To avoid exciting sR_{587} , we here observe the cells with light of wavelengths $>750 \text{ nm}$ using an infrared-sensitive camera and track the cells on a video monitor. This light is beyond the absorption range of sR_{587} and therefore no S_{373} is generated. Under these conditions, strong UV light which has been found previously¹² to be

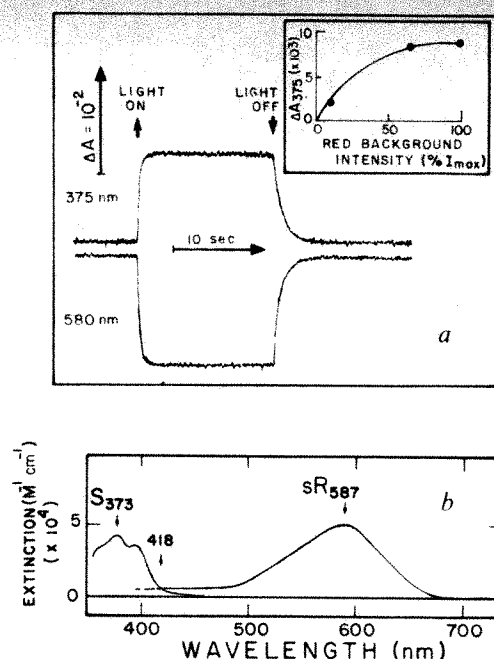


Fig. 2 *a*, Absorbance changes of sensory rhodopsin (sR) at 375 and 580 nm induced by red background light. The sample was a suspension of Flx3R vesicles, prepared vesicles, prepared and regenerated in a $3 \times 3 \text{ mm}$ quartz cuvette clear on four sides with all-*trans* retinal as described¹⁰. The regeneration of sensory rhodopsin was monitored as described¹⁰, use of the regenerated system permitting precise quantitation of the sensory rhodopsin content. Flx3 vesicles containing native sensory rhodopsin gave the same phototransients as the regenerated protein in Flx3R shown here. Flash-induced absorbance changes were measured as described⁹. Background excitation was provided by a tungsten halogen lamp beam passed through a long pass Corning 2-61 (cutoff 610 nm) and Ditic Optics 700 nm short pass filter combination and applied to the sample at 90° with respect to the measuring beam. The maximum red light intensity (I_{max}) was $4.25 \times 10^6 \text{ erg cm}^{-2} \text{ s}^{-1}$. The inset shows the light intensity-dependence of the absorbance changes at 375 nm (ΔA_{375}). *b*, Spectrum of S_{373} and sR_{587} . The latter is the spectrum obtained by regeneration of sensory rhodopsin apoprotein with all-*trans* retinal⁹ and the former was calculated by adding the sR_{587} spectrum to the light-induced difference spectrum obtained using the system described in *a*. The extinction coefficients are based on our measurements^{9,10} and have absolute uncertainty of $\pm 15\%$.

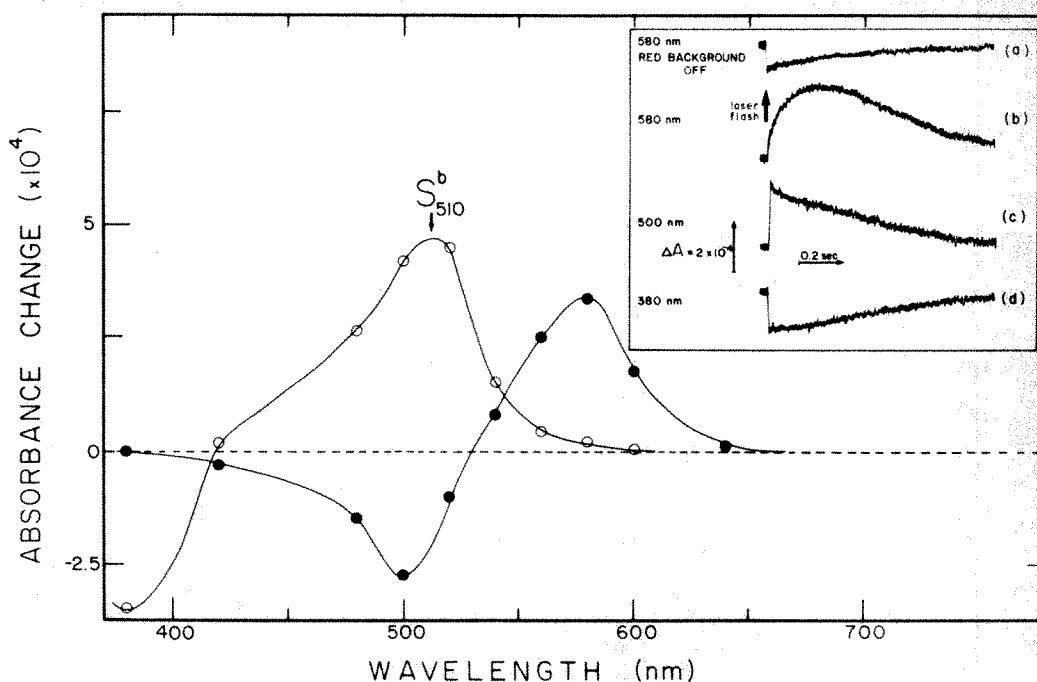
strongly repellent, causes no change in reversal probability (Fig. 1). Furthermore, addition of red background light which generates S_{373} sensitizes the cells to the repellent stimulus, causing nearly every cell to reverse swimming direction within 2 s (Fig. 1). We attribute this sensitization to the generation of a photostationary state containing the repellent receptor (S_{373}) by red light.

One might argue that the adaptation to the red stimulus lowered the threshold sensitivity to blue light because the background red light generated a transient attractant response when it was turned on. This possibility is ruled out because we show here that red light sensitizes the blue response even if it is delivered simultaneously with the blue stimulus.

Photochemical reactions

Because of its fast rise and slow decay, S_{373} accumulates in significant amounts in natural light absorbed by sR_{587} , producing a photostationary state mixture of the two species sR_{587} and S_{373} . The absorption spectra of these species, calculated from the photostationary state difference spectrum, are shown in Fig. 2. The spectra are sufficiently broad to permit laser photoexcitation of both species at 406 nm so that flash-induced absorbance changes at their regions of maximum absorption could be monitored with minimal interference from the intense actinic flash.

Fig. 3 Flash-induced absorbance changes of S_{373} . Inset, transient absorption changes of the sample shown in Fig. 2 in the absence (a) and in the presence (b-d) of 100% $I_{\max}$ red background illumination at three selected wavelengths. Actinic laser flash: 406 nm, 10 ns, 0.4 mJ. Main figure from traces shown in the inset and at other wavelengths the difference in the amplitude of absorbance change between 0 and 100 μ s ($\circ$) and between 200 ms and 100 μ s ($\bullet$) were calculated and plotted as functions of wavelength. Note that at 580 nm the ratio of the forward to back reaction absorbance change amplitude is the same as the ratio of absolute absorbances of sR_{587} and S_{373} at 406 nm (Fig. 2). This indicates that the photochemical quantum yields of the two reactions are nearly equal.



In the absence of background illumination, excitation at 406 nm causes a transient depletion of sR_{587} (Fig. 3a). This is due to the sR_{587} to S_{373} photoconversion as shown previously⁹. In the presence of saturating background illumination, which depletes the sample of sR_{587} (Fig. 2a), excitation causes a transient regeneration of sR_{587} from S_{373} (Fig. 3b, d). Absorbance changes measured at 500 nm (Fig. 3c) reveal an intermediate state in the S_{373} to sR_{587} reaction which forms in less than 50 μ s. Further analysis of flash kinetic data at other wavelengths (Fig. 3) shows that this intermediate state absorbs maximally near 510 nm and decays into sR_{587} with a half-time of about 80 ms. Absorption of red background light by sR_{587} regenerates S_{373} (Fig. 3d). Note that this establishes a cyclic reaction containing two photon absorption steps (Fig. 4).

Colour-sensing mechanism

Spectral discrimination by slow-cycling rhodopsin can be understood as an interplay between the two-photon cycle discussed above and the one-photon cycle generated by photoexcitation of sR_{587} and its subsequent thermal steps (Fig. 4). We propose that the one-photon reaction generates an attractant signal, transmitted to the flagellar motor by an attractant signal transduction pathway. Increases (decreases) in this signal suppress (induce) flagellar reversals. These effects occur in the absence of any background illumination. In contrast, the two-photon cycle generates a repellent signal, transmitted and amplified separately from the attractant signal, but integrated eventually before or at the site of action on the flagellar motor³. Increases (decreases) in this signal induce (suppress) flagellar reversals.

Test of the mechanism

Previous studies have shown that the cells are at least an order of magnitude more sensitive to repellent (blue) light than to attractant (red) light intensity changes^{2,3}. According to our interpretation this sensitivity difference must be conferred by amplification step(s) beyond the photoreceptor, because there can never be more repellent receptor molecules (S_{373}) than the maximum number of molecules available for attractant reception (sR_{587}) and their photochemical quantum yields are nearly equal (see Fig. 3 legend). Therefore the repellent signal from the two-photon cycle must be at least an order of magnitude more effective in inducing reversals than the one-photon cycle is in suppressing them. This leads to a seemingly paradoxical predic-

tion which is a strong test of the mechanism: we should be able to construct conditions in which an increase in red light will generate a repellent rather than the usual attractant response.

According to the proposed mechanism (Fig. 4), the repellent signal produced by S_{373} photoexcitation should be generated by any of three illumination patterns. First, photoexciting S_{373} previously generated by background red light (as in Fig. 1). This is the usual stimulus which makes blue light a repellent under natural illumination.

Second, imposing an intense background of blue illumination which, in the absence of red light, will not influence the cells' swimming behaviour because slow-cycling rhodopsin is almost completely in the sR_{587} form, followed by stimulation with red light. The S_{373} thereby generated will undergo the two-photon cycle, which should cause the repellent rather than the attractant response. This condition is realized in Fig. 5 where a given increase in red light was delivered in the absence and in the presence of blue background illumination. A clear suppression

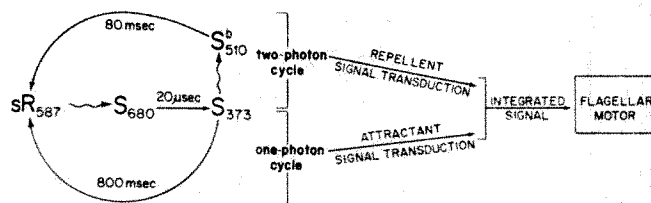


Fig. 4 Mechanism of colour discrimination in *H. halobium* phototaxis. Photon absorption by sR_{587} , the dark-adapted form of sensory rhodopsin, generates a short-lived species S_{680} which rapidly decays to S_{373} . The S_{373} form can return to the sR_{587} form by either of two paths: a purely thermal relaxation or a photoinduced path. If S_{373} does not absorb a photon, it relaxes thermally to sR_{587} completing the 'one-photon' photocycle. During its transit through the one-photon cycle, the sensory rhodopsin molecule generates an attractant signal to the flagellar motor. If S_{373} absorbs a photon, it generates the intermediate S_{510} . The relaxation of S_{510} to sR_{587} completes a cycle containing two photon absorption steps. After absorption of the second photon in the 'two-photon' cycle, sensory rhodopsin generates a repellent signal to the flagellar motor. The attractant and repellent signals are integrated prior to or at their site of action on the flagellar motor³.

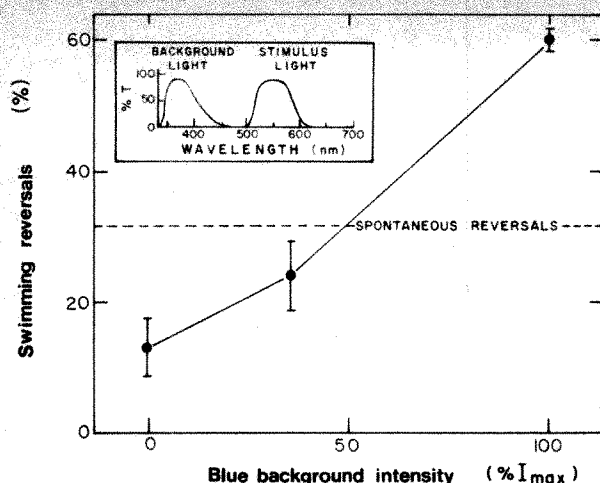


Fig. 5 Effect of near ultraviolet background light on cell behavioural response to red light. Experimental conditions as described in Fig. 1 except that the response was assayed as the % reversals within 3 s of a red light stimulus. The range of spontaneous reversal frequencies of populations used in these studies was $14\% \pm 7\%$ reversing within 2 s. All effects of background illumination on behaviour reported here and in Figs 2 and 6 have been confirmed in populations with both high and low spontaneous reversal frequencies.

of spontaneous reversals occurs in the absence of background blue as expected from the one-photon cycle attractant signal. The dramatic result is that the same red light stimulus elicits a repellent (reversal-inducing) response in the presence of the highest blue light intensity.

Third, delivering both red and blue lights simultaneously at the same intensities as Fig. 5. Again the repellent response should result since it makes no difference whether the blue light was present prior to the generation of S_{373} .

All the permutations of blue and red light stimuli are shown in Fig. 6. Our results can be interpreted in terms of the proposed model. Figure 6a gives the spontaneous reversal frequency of 22%, which is not affected significantly by blue alone. Figure 6b shows 16% reversals, because there is essentially no S_{373} (some S_{373} will be generated by the weak sR_{587} absorption in the blue, but calculations show the amount of S_{373} formed is so little that no two-photon cycling will occur in the time of the measurement). The three illumination patterns discussed above are shown by Fig. 6c, e and f respectively, all inducing an almost complete reversal response. Red light alone significantly suppresses reversals (Fig. 6d).

Conclusions

The experiments we reported previously strongly implicated slow-cycling rhodopsin as the attractant receptor for *H. halobium* phototaxis^{9,10}. The evidence presented here implicates this protein as the repellent receptor as well. We prove here that, like S_{373} , the repellent receptor is a photoproduct of a slow-cycling, red-absorbing, retinal pigment with the properties of sR_{587} .

From these experiments we propose a mechanism in which slow-cycling rhodopsin has two signal transducing forms, one transmitting an attractant signal and the other a repellent signal to the flagellar motor (Fig. 4). Increases in the repellent signal or decreases in the attractant signal induce flagellar reversals. Conversely, decreases in the repellent signal or increases in the attractant signal suppress flagellar reversals. Both induction and suppression of reversals are transient responses, demonstrating adaptation of the cell to the signals. The net effect is that the motility apparatus of the cell responds to changes in the numbers of slow-cycling rhodopsin molecules undergoing the one-photon and two-photon cycles. In view of these results, we suggest the original name⁹ 'slow-cycling rhodopsinlike pigment' be changed

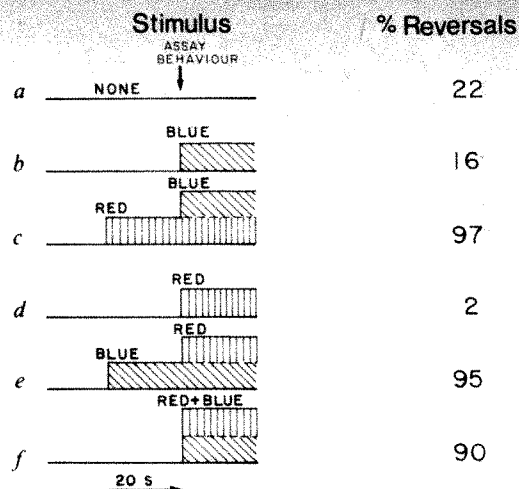


Fig. 6 Behavioural response under various permutations of stimulus and background light. Values are averages of duplicate measurements which vary by less than 15%. Experimental conditions as described in Fig. 1 except that reversals were assayed within 3 s of the stimulus, blue and red light were delivered using a tungsten light source and wavelengths were selected by adding or removing filters from the single beam. The Hg light source was blocked in this experiment. 'Blue', 380–433 nm (Optics Technology 433 short pass filter + 2 mm Plexiglass + two 1–75 Corning infrared absorbing glass), intensity $3.3 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$. 'Red', 530–700 nm (Corning 3–68 + two Corning 1–75), intensity $1.0 \times 10^5 \text{ erg cm}^{-2} \text{ s}^{-1}$. 'Red + Blue', The tungsten beam filtered through two Corning 1–75 + 2 mm Plexiglass, intensity $1.5 \times 10^5 \text{ erg cm}^{-2} \text{ s}^{-1}$.

to 'sensory rhodopsin' of *H. halobium*, reflecting more clearly the physiological function of the molecule.

The absorption spectrum of S_{373} matches closely the $\sim 370 \text{ nm}$ action spectrum peak reported for phototaxis repulsion^{2,4,5}. However, the absorption spectrum of sR_{587} matches some but not all of the published action spectra for phototaxis attraction. The attractant response was reported to have a single peak at $\sim 565 \text{ nm}$ in wild-type^{2,5} and two peaks, one at $\sim 565 \text{ nm}$ and the other at $\sim 590 \text{ nm}$, near the sR_{587} absorption maximum in a bacteriorhodopsin-deficient strain¹². The disagreement may be caused by the presence of bacteriorhodopsin and halorhodopsin which may influence the motility response directly or indirectly.

Dencher¹³ and Hildebrand and Schimz¹⁴ have proposed that two distinct receptors mediate the attractant and repellent sensitivities. Our data prove that the retinal-dependent repellent receptor is a photoproduct of a red absorbing pigment. Therefore their proposal requires the existence of at least one additional hypothetical red-absorbing retinal pigment. Our model requires only the single identified slow-cycling rhodopsin and, moreover, makes several specific quantitative predictions: (1) a delayed blue flash following a red flash will change from an attractant to a repellent stimulus with delay time predicted by the $20 \mu\text{s}$ rise time of S_{373} ; (2) mutants deficient in slow-cycling rhodopsin would be predicted to lose both attractant and repellent photosensitivities, and reversion to normal phototaxis behaviour should be accompanied by return of the pigment.

A remarkable finding from our measurements is that photoexcitation of a single molecule of S_{373} appears to be sufficient to generate a reversal-inducing repellent signal to the flagellar motor. From the measured intensity ($3.6 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$) and spectrum of the background 'red' illumination (Fig. 1), the sR_{587} and S_{373} molar extinction spectra (Fig. 2b) and the parameters of the photocycle (Fig. 4), we calculate 2% of the sensory rhodopsin molecules will be in the S_{373} form in the photostationary state at 50% maximal intensity for red background (Fig. 1). From the regeneration of sensory rhodopsin absorption by retinal (see Fig. 2 legend) and the sensory rhodopsin extinction ($54,000 \text{ M}^{-1} \text{ cm}^{-1}$) at that wavelength, we measure 5×10^3

molecules per cell, ~ 100 of which therefore will be in the S_{373} form. From the quantum flux of blue light used for the stimulus (Fig. 1) and the measured S_{373} extinction in the stimulus spectral range (Fig. 2b), we calculate the S_{373} population in the average cell will absorb only about two photons in the 2-s period over which reversals were assessed. Taking the quantum efficiency for photochemical reaction to be 0.5 ± 0.2 , covering the range of all known retinal pigments, only ~ 0.7 – ~ 1.4 S_{373} molecules per cell on average would be activated photochemically by a stimulus which causes over 50% of the cells to respond. Therefore photoexcitation of a single S_{373} molecule appears to be sufficient to alter the cell's swimming behaviour.

The cell is at least an order of magnitude less sensitive to attractant than repellent light^{2,3}. In our interpretation this means the amplification of the S_{373} signal by the signal transduction machinery (Fig. 4) is at least $10\times$ greater than that of sR_{587} . The relative amplification in the two signalling pathways may depend on the specific conditions and stage of cell culture growth (unpublished observations).

The colour-sensing mechanism described here depends on the existence of two spectrally-distinct signalling forms of the same receptor. This could be a unique property of the sensory rhodopsin of halobacteria. Alternatively, the sensory rhodopsin mechanism may reflect an inherent potential of retinal/protein

complexes—photoreversibility of photoconversion products of the retinal/opsin chromophore—important to the evolution of the rhodopsin family as photosensory receptors. Even retinal pigment photoproducts which are too short-lived to accumulate under physiological conditions (such as the K, L and M intermediates formed from bacteriorhodopsin⁶ and the early photoproducts formed from mammalian rod rhodopsin¹⁴) are photoreversible. Invertebrate rhodopsins, like sensory rhodopsin and the non-retinal plant chromoprotein phytochrome, are photochromic; that is, they have photoreactions with thermal relaxation sufficiently slow to cause significant photostationary state accumulation of their photoreversible conformations¹⁶. The sensory rhodopsin mechanism, preserved in the archaeobacterium *H. halobium*, may have provided a primitive colour-sensing capability. In the evolution of visual systems, this mechanism may have been maintained in some organisms but replaced in others by multiple receptor mechanisms with increased colour resolution.

We thank W. Stoeckenius, L. Stryer, E. Spudich and S. Sundberg for critical reading of the manuscript and J. Korenbrot for use of his infrared-sensitive video equipment. This work was supported by NIH grant GM27750 and an Irma T. Hirsch Trust Career Scientist Award (J.L.S.); NIH grant GM27057, NASA grant NSG-7151 and NSF grant PCM-8316139 (R.A.B.).

Received 17 May; accepted 28 September 1984.

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The *neu* oncogene: an *erb-B*-related gene encoding a 185,000- M_r tumour antigen

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A series of rat neuro/glioblastomas all contain the same transforming gene (neu) which induces synthesis of a tumour antigen of relative molecular mass (M_r) 185,000 (p185). The neu oncogene bears homology to erb-B and the tumour antigen, p185, is serologically related to the epidermal growth factor (EGF) receptor. The two proteins, EGF receptor and p185 appear to be distinct, as they coexist in nontransformed Rat-1 cells.

GENE transfer (transfection) has revealed the presence of oncogenes in the genomes of a variety of spontaneous and chemically induced tumours. Many of these oncogenes are allelic versions of one of the three genes belonging to the *ras* gene family. In several cases, the functional differences between these oncogenes and their cognate proto-oncogenes derive from single nucleotide changes, mutations which apparently have occurred in the target tissue as one of the steps of carcinogenesis (reviewed in refs 1, 2).

We have been investigating a family of transforming genes in the genomes of several rat neuro/glioblastoma cell lines derived from tumours induced by ethylnitrosourea (ENU)³. Injection of pregnant BDIX rats with this carcinogen during day 15 of gestation results in the appearance of neuro/glioblastomas in

the offspring. DNA prepared from four neuro/glioblastoma cell lines transforms NIH 3T3 cells when introduced by transfection⁴, but Southern blot analysis of the genomes of the transformed cells gave no indication of the presence of acquired *ras* oncogenes (A.L.S. and L.V., unpublished results), suggesting that a *ras* gene was not involved in the transforming activity.

The four independently activated neuro/glioblastoma oncogenes exhibited a common pattern of sensitivity or resistance to inactivation by restriction enzyme cleavage (C. Shih and R.A.W., unpublished data), suggesting that all are allelic versions of a common antecedent gene. We have applied the term '*neu*' to this group of apparently related genes.

These neuro/glioblastoma oncogenes are associated with the expression of a common tumour antigen⁵. NIH 3T3 cells

transformed by *neu* oncogenes were used to seed tumours in young mice, the sera from which precipitated a phosphoprotein of relative molecular mass (M_r) 185,000 (185K) from lysates of all NIH 3T3 lines that carry the neuro/glioblastoma oncogenes. This protein, designated p185, was detected neither in untransformed NIH 3T3 cells nor in NIH cells transformed by various *ras* oncogenes. Furthermore, when *ras*-transformed NIH 3T3 cells were used to seed tumours in mice, no serum reactivity to p185 was induced. Thus, synthesis of p185 is strongly associated with the presence of the neuro/glioblastoma oncogenes⁵.

Results from several laboratories have shown that the cellular homologues of certain oncogenes first identified in retroviral genomes are also important in the genesis of spontaneous and chemically induced tumours (reviewed in refs 1, 2). One of these retrovirus-associated oncogenes is *erb-B*, which was recently shown to be homologous with the cellular gene encoding the epidermal growth factor (EGF) receptor⁶. The EGF receptor and p185 have similar molecular weights, and they are both phosphorylated cell-surface glycoproteins (refs 7-11 and A.L.S., D.F.S. and R. Negrin, unpublished data). Thus, we suspected a possible relationship between *erb-B* and the *neu* oncogenes. We report here on the homology of the *neu* and *erb-B* genes and on the relationship of p185 to an *erb-B* gene product, the EGF receptor.

Homology with *erb-B*

Possible homology between *erb-B* and *neu* was tested by Southern blot hybridization. DNA was prepared from a panel of NIH 3T3 cells, each of which had been transformed by DNA from one of the rat neuro/glioblastoma cell lines. The DNAs were digested with endonuclease *Eco*RI before electrophoresis. Figure 1c-p shows that NIH 3T3 cells that have been transformed by the neuro/glioblastoma DNA carry an *Eco*RI fragment that reacts with an *erb-B*-specific sequence probe. The variability in size of the new *Eco*RI fragments probably results from rearrangements in sequences flanking the transforming gene which occur during transfection. Such variability has been observed previously in cells transfected with the EJ bladder carcinoma oncogene¹². An *erb-B*-homologous fragment of similar size is present in much smaller amounts in untransformed NIH 3T3 cells (Fig. 1q).

To distinguish between the rat and mouse *erb-B* homologues and to ensure that the large *Eco*RI segments shown in Fig. 1c-p

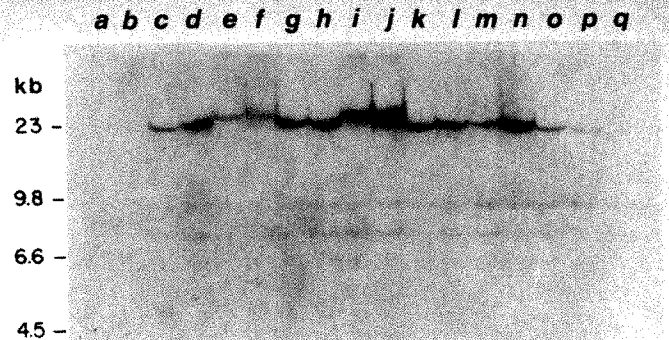


Fig. 1 Southern blot analysis of *erb-B*-related sequences in NIH 3T3 cells transformed with rat neuro/glioblastoma DNAs; *Eco*RI digestion. The DNAs analysed were obtained from the following: a, BDIX rat liver; b, B104; c, d, primary transfectants of B104 (B104-1 and B104-3); e, f, secondary transfectants of B104 (B104-1-1 and B104-1-2); g, h, primary transfectants of B103 (B103-5 and B103-1); i, j, secondary transfectants of B103 (B103-1-1 and B103-1-2); k, B50-1, a primary transfectant of B50; l, m, secondary transfectants of B50 (B50 6a-2 and B50 6a-3); n, o, primary transfectants of B82 (B82-1 and B82-3); p, B82-3-2, a secondary transfectant of B82; q, NIH 3T3. M, standards comprising λ phage *Hind*III restriction fragments are shown on the left.

Methods: High-molecular weight DNA (10 μ g) was digested with endonuclease *Eco*RI, fractionated by electrophoresis in 0.9% agarose gels, and transferred to nitrocellulose paper. The filters were hybridized at low stringency (30% formamide, 5 $\times$ SSCPE, 42 $^{\circ}$ C) for 36 h with an *erb* specific probe (a *Sac*I-*Pvu*II 1.8-kb subclone of avian erythroblastosis virus²²) nick-translated with ³²P-dCTP. The filters were then washed three times at 20 $^{\circ}$ C for 10 min and for 5 h at 50 $^{\circ}$ C in 2 $\times$ SSCPE. The filters were exposed to Kodak X-Omat AR for 3 days at -70 $^{\circ}$ C.

are of donor rat origin, we treated the DNA of the transfected cells with endonucleases *Eco*RI and *Bam*HI before Southern blot analysis. This analysis confirmed that the transfected cells carry an acquired 4.4-kilobase(kb) *BAM*/RI DNA segment which has no counterpart in the genome of the recipient mouse cells (Fig. 2). Thus, 14 independently transfected cell lines shown to express high levels of p185 have acquired a DNA segment exhibiting some homology with the *erb-B* probe. Because these

Table 1 Immunofluorescent staining of rat and human cell lines by antibody 7.16.4

Cell lines	Description	Immunofluorescence
B104	<i>neu</i> ⁺ rat neuroblastoma	+
B82	<i>neu</i> ⁺ rat neuroblastoma	+
B50	<i>neu</i> ⁺ rat neuroblastoma	+
B15	<i>neu</i> ⁻ rat neuroblastoma	+++
B65	<i>neu</i> ⁻ rat neuroblastoma	++
B104-1-1	NIH 3T3 transfectant of <i>neu</i> ⁺ DNA	++++
NIH 3T3	Mouse fibroblast cell line	-
Rat(B104)-2	Rat-1 transfectant of <i>neu</i> ⁺ DNA	++++
Rat(B104)-3	Rat-1 transfectant of <i>neu</i> ⁺ DNA	+++
Rat(B104)-4	Rat-1 transfectant of <i>neu</i> ⁺ DNA	+++
Rat-1	Rat fibroblast cell line	+
WTCL4	Rous sarcoma virus-induced rat neuroblastoma	++
A431	Human cervical carcinoma line	-
PC3	Human prostate carcinoma line	-
MCF7	Human breast carcinoma line	-
EJ	Human bladder carcinoma line	-
G361	Human colon carcinoma line	-
HI135	Human melanoma line	-

Cell lines were prepared for FACS analysis on a Coulter Epics V Cell Sorter as described in ref. 11. Immunofluorescence was quantified by subtracting the median fluorescence channel of populations stained with FITC-conjugated rabbit anti-mouse immunoglobulin alone (negative control) from the median fluorescence channel of populations stained with monoclonal antibody 7.16.4 followed by FITC-conjugated rabbit anti-mouse (positive staining). The resulting net fluorescence channel was expressed according to the following scale: -, less than two channels; +, 5-10 channels; ++, 11-15 channels; +++, 16-20 channels; +++++, >20 channels.

cell lines derive from several independent transfections of four different neuro/glioblastoma tumour DNAs, we concluded, as we had speculated, that a common oncogene is present in the genomes of the four donor neuro/glioblastomas.

The *erb-B* oncogene probe reacts with at least two fragments produced by *Eco*RI cleavage of rat DNA (Fig. 1a, b) which are respectively 7 and 34 kb long (M.-C. Hung and A.L.S., unpublished results). The 34-kb fragment is of identical size to the rat DNA segment found in transfected NIH 3T3 cells and must represent the normal counterpart to the DNA segment bearing the *neu* oncogenes. Because *Eco*RI does not inactivate the *neu* oncogene (C. Shih and R.A.W., unpublished results), we conclude that this oncogene lies entirely within the 34-kb *Eco*RI fragment. The 7-kb *erb-B*-related DNA segment may represent a distinct but related gene. The Southern blot analysis does not indicate which of these *erb-B*-homologous segments encodes the EGF receptor, so that we could not confirm whether the *neu* gene is identical with, or only related to, the gene encoding the EGF receptor.

p185 and EGF receptor are related

The homology between *erb-B* and *neu* suggested that the EGF receptor, a known *erb-B* gene product, might be related to p185, the protein induced in cells transfected with *neu*. Two different antibody preparations were used to examine this possibility. Monoclonal antibody 7.16.4 was derived from mice immunized with B104-1-1 cells (a cell line derived by two serial passages of the *neu* oncogene through NIH 3T3 cells; ref. 4). The reactivities of the 7.16.4 antibody are indicated by the immunofluorescence data shown in Table 1. The antibody recognizes a cell-surface determinant displayed by rat neuro/glioblastomas, by a variety of normal and transformed rat cell lines and by NIH 3T3 cells transformed by the rat oncogene; it does not react with normal NIH 3T3 cells or with a variety of human tumour cell types, including the A431 cells known^{13,14} to display large amounts of EGF receptor. The 7.16.4 antibody appears to react with a rat-specific determinant of p185. Further characterization of this antibody is reported elsewhere¹¹. The second source of antibody was polyclonal anti-EGF receptor serum raised against EGF receptor purified from human A431 cells; this serum reacts with human and rodent EGF receptor¹⁵.

We used these sera to elucidate the relationship between the EGF receptor and p185. Lysates of B104-1-1 cells, metabolically labelled with ³⁵S-cysteine, were incubated with normal mouse serum, serum 7.16.4 or anti-EGF receptor serum; p185 was precipitated by the 7.16.4 serum (Fig. 3h), but not by the control serum (Fig. 3g). Incubation with anti-EGF receptor serum (Fig. 3i) yielded a protein that co-migrated with p185. This suggested that the p185 induced by the rat *neu* oncogene shares antigenic determinants with the human EGF receptor.

To confirm that the two immune sera recognize the same protein in B104-1-1 cells, labelled lysates cleared by incubation with one antibody were subsequently reacted with the other. For example, the supernatant remaining after incubation with antibody 7.16.4 was then reacted with the anti-EGF receptor serum, which did not precipitate a 185K protein from the lysate (Fig. 3l). However, anti-EGF receptor did precipitate the 185K protein from a lysate cleared with normal serum (Fig. 3k). Similarly, p185 could be precipitated by 7.16.4 serum from a lysate that had been cleared with normal serum (Fig. 3m), but not from a lysate that had been cleared with anti-EGF receptor serum (Fig. 3n). (The decreased intensity of specific and non-specific bands in lanes k to n of Fig. 3 compared with lanes g to j indicates that nonspecific losses occurred during the second round of immunoprecipitation.) These results confirm that an anti-EGF receptor serum reacts with p185. Thus, we conclude that p185 and EGF receptor have antigenic determinants in common.

Although p185 and EGF receptor are serologically related, they have different electrophoretic mobilities. The rat EGF receptor has an apparent M_r of ~170,000, while p185 is some-

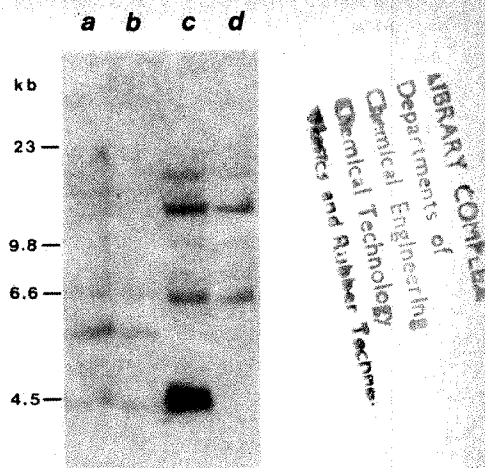


Fig. 2 Southern blot analysis of *erb-B*-related sequences; *Eco*RI plus *Bam*HI digestion. The DNAs analysed were: a, BDIX rat liver; b, B104; c, B104-1; d, NIH 3T3. High-molecular weight DNA (10 µg) was digested with restriction endonucleases *Eco*RI and *Bam*HI and analysed as described in Fig. 1 legend.

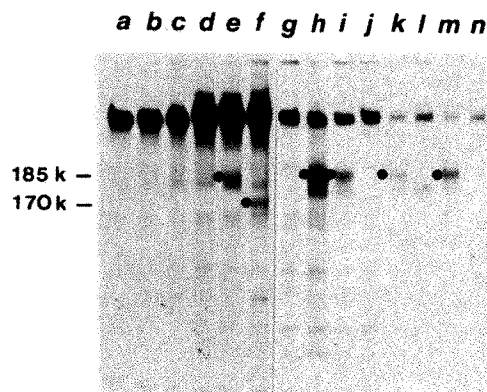


Fig. 3 Immunoprecipitation of p185 and EGF receptor. Lysates of ³⁵S-cysteine-labelled cells were incubated with antibody and subsequently precipitated. Lane a, NIH 3T3 cells incubated with normal mouse serum (NMS); b, NIH 3T3 cells incubated with antibody 7.16.4; c, NIH 3T3 cells incubated with anti-EGF receptor antiserum; d, Rat-1 cells incubated with NMS; e, Rat-1 incubated with antibody 7.16.4; f, Rat-1 incubated with anti-EGF receptor serum. g-n, B104-1-1 incubated with the following: g, NMS; h, antibody 7.16.4; i, anti-EGF receptor serum; j, NMS; k, supernatant from g, incubated with anti-EGF receptor; l, supernatant from h, incubated with anti-EGF receptor; m, supernatant from j, incubated with antibody 7.16.4; n, supernatant from i, incubated with antibody 7.16.4.

Methods: 3×10^6 cells (B104-1-1) or 3×10^5 cells (NIH 3T3 and Rat-1) were seeded in 60-mm culture dishes and labelled for 18 h in 0.8 ml of Hank's minimal essential medium containing 0.1 the usual amount of cysteine, 2% dialysed fetal calf serum, and 250 µCi ³⁵S-cysteine (977 Ci mmol⁻¹; NEN) per ml. Cells were lysed in phosphate-buffered RIPA buffer containing 1 mM ATP, 2 mM EDTA and 20 mM sodium fluoride and immunoprecipitates prepared and washed as described elsewhere²³. Portions of the lysates were incubated with NMS (1 µl), antibody 7.16.4 (concentrated, 1 µl) or 3 µl anti-EGF receptor antiserum for 60 min, then with 1 µl of sheep anti-mouse immunoglobulin (Cappel). Incubation was continued for 30 min, after which immunoprecipitates were collected using fixed *Staphylococcus aureus*. Precipitates were prepared from 300,000 cells (lanes a-f), 60,000 cells (g, h, k and l) or 30,000 cells (i, j, m and n). (Samples in g-n were all derived from the same original lysate.) Precipitates in lanes k and l were prepared from supernatants remaining after precipitation of the lysates shown in lanes g and h. Precipitates in lanes m and n were prepared from lysates first precipitated to yield the samples shown in lanes j and i, respectively. The samples were analysed by SDS-polyacrylamide gel electrophoresis in a 7.5% acrylamide-0.17% bisacrylamide gel. The gel was fluorographed²⁴ and exposed to preflashed film for 7 days at -70 °C.

what larger; this difference could arise from two sources. It is possible that the mutation(s) leading to activation of the *neu* oncogene grossly affect the structure of the encoded protein so that a gene normally encoding an EGF receptor-sized protein might, after oncogenic activation, encode a protein the size of p185. Alternatively, the *neu* oncogenes might derive from a distinct proto-oncogene that normally encodes a 185K protein.

To distinguish between these possibilities, we examined two nontransformed fibroblast cell lines, hoping to identify possible normal protein homologues of p185. We have not yet been able to identify large proteins specifically precipitated from NIH 3T3 cells by either antibody 7.16.4 or anti-EGF receptor serum (Fig. 3a-c). However, these antibodies do recognize proteins present at low levels in untransformed Rat-1 cells. The anti-EGF receptor preferentially precipitates a protein of 170K (Fig. 3f) which has a slightly greater mobility than the EGF receptor precipitated from A431 cells (D.F.S., unpublished results) and is probably the rat EGF receptor. In contrast, antibody 7.16.4 precipitates a protein (Fig. 3e) that migrates slightly more rapidly than p185 (lanes h, i), but this antibody does not precipitate the putative EGF receptor. These data indicate that the two sera recognize distinct proteins in Rat-1 cells, and support the notion that p185, induced by the *neu* oncogene, stems from a protein of similar size present in normal cells. The normal protein may be distinct from the EGF receptor.

Implications

The *neu* gene was found to be activated in cell lines derived from four of six independent rat neuro/glioblastomas, the DNAs of which were tested by transfection⁶. Thus, *neu* activation is a frequent occurrence when the ENU pulse carcinogenesis protocol is used to induce neuroectodermal tumours in rats. Induction of tumours in rodents by defined chemical or radiological regimes has, in other instances, resulted in reproducible activation of particular oncogenes^{16,17}, but, hitherto, only *ras* gene activation has been reported.

The activation of the *neu* oncogenes in ENU-treated rats is of special interest because this experimental model of carcinogenesis has been characterized in great detail. These neuro/glioblastomas are induced by exposure to ENU during a well-defined window of time in late fetal and early postnatal development. Studies by Rajewsky and co-workers indicate that proliferation of target blast cells and the absence of a DNA repair system in embryonic rat brain cells strongly influence the kinetics of tumour induction¹⁸. Moreover, these investigators have developed an *in vitro* culture system in which brain cells are explanted from fetal rats following transplacental mutagenesis with ENU. These cultures undergo a series of changes and ultimately yield tumorigenic clones with the same lag period as that required for induction of tumours *in vivo*¹⁹. By using this culture system, it may be possible to determine whether *neu* activation is an early or late event in tumorigenesis, and whether early identification of premalignant cells is possible using p185-specific antibodies.

We have found that there is nucleic acid sequence homology between *neu* and *erb-B* oncogenes, and that the EGF receptor,

encoded by an *erb-B*-related gene, is antigenically related to the *neu*-associated tumour antigen, p185. These results strongly suggest that p185 is a product of the *neu* oncogene.

The *erb-B* probe recognizes at least two DNA fragments in the rat genome, one of which encompasses a biologically active *neu* oncogene. This raises the possibility that there may be more than one functional *erb-B*-related gene in the rat genome, and that the gene which specifies EGF receptor is partially or completely separate from the *neu* proto-oncogene.

The data of Fig. 2 indicate that the anti-EGF receptor and 7.16.4 antibodies recognize distinct proteins that coexist in non-transformed Rat-1 cells. This suggests that the differences between the EGF receptor and a p185-like protein can be observed in cells that have not undergone oncogenic transformation. We consider it possible that the two proteins, while related to one another in structure, are made on distinct templates within the same cell and derive, at least in part, from distinct sequences in the DNA. Further structural characterization of the two proteins is under way.

Whether *neu* is an allele of the gene that encodes EGF receptor or a second related gene, the similarities between p185 and EGF receptor suggest that the normal *neu* gene product is a growth factor receptor. We speculate that the *neu* transforming protein functions by delivering a mitogenic signal to the interior of the cell, even in the absence of appropriate stimulation by the cognate growth factor—this would be analogous to the finding that the *v-erb-B* gene product lacks the EGF-binding domain of the receptor and must therefore be insensitive to the presence of EGF⁶.

The role of growth factor receptors in spontaneous tumorigenesis was previously inferred from the findings that a human vulval carcinoma cell line (A431)^{13,14}, human squamous cell carcinomas²⁰ and human glial tumours²¹ have large numbers of surface EGF receptors. Taken together with our findings, these results indicate that activation of growth factor receptors may be a frequent occurrence in tumorigenesis. Unlike the *ras* gene product, polypeptide growth factor receptors possess extracellular domains. It is possible that altered growth factor receptors bear novel tumour-specific antigens that can be identified by monospecific antisera. In contrast to most tumour-associated surface antigens, which are probably incidental to the transformation of a cell, the expression of p185 and analogous proteins seems to be intrinsic to the establishment and maintenance of the transformed state. This may make it possible specifically to alter the growth properties of these cells with immunological reagents that recognize determinants expressed on their surface.

We thank P. Heffernan and V. Link for technical help, and M.-C. Hung and M. Rosner for useful discussions. A.L.S. is supported by a US NIH postdoctoral fellowship. D.F.S. was supported by the Damon Runyon Cancer Fund and the US NIH. S.J.D. was supported by USPHS grant GM13972 from the NIH. J.A.D. was supported by an NIH grant from the Medical Scientist training program at Harvard Medical School. Work in the laboratory of R.A.W. was supported by grants CA31649 and CA26717 from the US NCI and by a grant from the American Business Cancer Research Foundation.

Received 18 June; accepted 19 September 1984.

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Allelic exclusion and control of endogenous immunoglobulin gene rearrangement in κ transgenic mice

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Hybridomas were produced from spleen cells of κ transgenic mice to investigate expression of the transgenic κ gene, its effect on allelic exclusion and its effect on the control of light-chain gene rearrangement and expression. Our results show that the transgene is expressed normally and that the production of a complete immunoglobulin molecule turns off light-chain gene rearrangement.

B LYMPHOCYTES must correctly juxtapose three heavy-chain gene segments¹ V_H , D_H and J_HC_H , and two light-chain gene segments² V_L and J_LC_L , in order to produce the two identical heavy polypeptides and two identical light chains that assemble into a complete immunoglobulin molecule. B lymphocytes exhibit allelic and isotypic exclusion—out of two possible heavy-chain alleles and eight possible light-chain alleles (two κ and six λ), a single B cell expresses only one heavy chain together with one light chain³. We seek here to distinguish between two suggested mechanisms for allelic exclusion. A stochastic model assumes that the chance of two functional rearrangements of a heavy- or light-chain gene in the same B cell may be so low that the outcome is exclusion of the other alleles and isotypes in the majority of B cells, because rearrangement is highly error-prone⁴⁻¹³. Another postulated mechanism involves a direct feedback from a functionally rearranged gene or its protein product which shuts off further immunoglobulin gene rearrangements¹⁴⁻¹⁶. Details of the possible mechanisms for allelic exclusion have been widely discussed^{4,14-17}.

To determine whether a functionally rearranged light-chain gene influences other immunoglobulin gene rearrangements, we have microinjected a functional κ gene (cloned from the myeloma MOPC-21) into fertilized mouse eggs and produced κ transgenic mice^{18,19}. A functionally rearranged κ gene is therefore present in all B cells of these mice before any endogenous immunoglobulin genes begin to rearrange. In the present work we made hybridomas from spleen cells of these transgenic mice to investigate the expression of the κ -transgene on a single cell level, its effect on allelic exclusion and its effect on the control of light-chain gene rearrangement.

Hybridoma immunoglobulin

Hybridomas were produced from the spleens of three different transgenic mice. We first tested hybridomas for pBR322 DNA (included on the microinjected κ DNA to serve as a marker for the presence of the injected gene) (data not shown) and selected for further analysis 24 clones retaining the microinjected gene and two clones that had lost the chromosome bearing the microinjected gene. Based on secreted κ chains, three types of hybridoma are demonstrated (Fig. 1): cells which secrete the transgenic κ chain, cells which secrete an endogenous κ chain and cells which secrete no κ chain. Of 20 secreting hybridomas, none was found which secretes both transgenic and endogenous κ chains. The transgenic κ secretors (Fig. 1, lanes 17–20) secrete the transgenic κ chain and have only this κ chain in their cytoplasm. The endogenous κ secretors secrete κ chains having isoelectric points different from the MOPC21 κ chain and from each other (lanes 9–16); they can be divided further into two groups on the basis of the presence (lanes 14 and 16) or absence (lanes 10 and 12) of the transgenic κ chain in the cytoplasm. The non-secretors (four hybridomas) secrete no κ chains but have traces of transgenic κ chains in the cytoplasm (lanes 5–8).

Because the samples in Fig. 1 were reduced, we could not eliminate the possibility that the hybridomas are secreting free light chains. The primary structure of immunoglobulin light chains controls their secretion²⁰. Indeed, a single amino acid substitution in the variable region can prevent secretion of free light chain²¹. We have used a property of the MOPC-21 light chain which is not secreted as free light chain but is readily secreted in association with heavy chains²². When the proteins

Table 1 Summary of hybridomas

Type	Hybridoma sample size	Cytoplasmic κ chains		Secreted κ chains		pBR322 DNA	Endogenous κ genes rearranged	Heavy chain mRNA		Heavy chain protein		No. tested for heavy-chain protein	Total
		T	E	T	E			μ	γ	μ	γ		
Transgenic κ_H secretors	1 from mouse 48 3 from 54 7 from 55	+	—	+	—	+	—	4	7	4	6	10/11	11
Endogenous κ_H secretors	1 from 48 1 from 55	—	+	—	+	+	+	2	0	2	0	2/2	2
Endogenous free κ secretors	2 from 48 4 from 54 1 from 55	+	+	—	+	+	+	5	2	—	—	7/7	7
Non-secretors	1 from 54 3 from 55	+	—	—	—	+	2+ 2—	2	2	—	—	4/4	4
Controls	1 from 48 1 from 54	—	—	—	—	—	+	1	1	—	—	2/2	2

Summary of the different kinds and numbers of hybridomas analysed from κ transgenic mice. Cytoplasmic and secreted κ chains were analysed as described in legends to Figs 1, 2; RNA as described in the text. The presence of pBR322 DNA was detected by preparing DNA from hybridomas, dotting onto nitrocellulose and hybridizing with radioactively labelled pBR322 (data not shown).

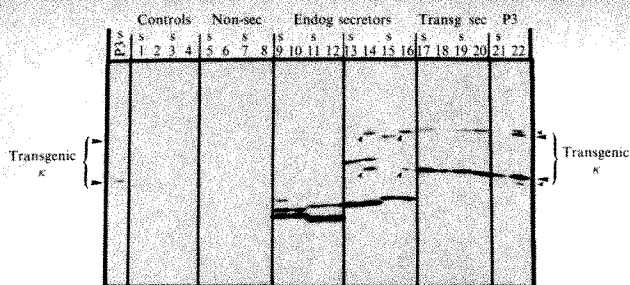


Fig. 1 Isoelectric focusing protein blot of cytoplasmic and secreted κ chains from transgenic hybridomas. Each box of four lanes contains secreted and cytoplasmic κ chains from two hybridomas that represent a particular type. Even-numbered lanes are cytoplasmic proteins; odd-numbered lanes are hybridoma supernatants (S). Lanes P3, 21 and 22 contain the P-3 cell line, a derivative of MOPC-21 that expresses the V_{κ} M.21 light chains. Lanes 1, 2, fusing line SP2/0, producing no κ chain; lanes 3, 4, a control hybridoma that lost the microinjected gene and secretes no κ chains; lanes 5–8, two non-secretors; lanes 9–12, two endogenous κ secretors having no cytoplasmic transgenic κ chains; lanes 13–16, two endogenous κ secretors that have cytoplasmic transgenic κ chains (1) but no secreted transgenic κ chains; lanes 17–20, two transgenic κ secretors.

Methods: Because we needed to use hybridoma technology to isolate a representative B-cell population from the spleens of transgenic mice, three different transgenic mice (numbers 48, 54 and 55; refs 18, 19) were immunized with the polyclonal activators lipopolysaccharide, LPS (5.0 μ g) or sheep red blood cells (0.2 ml of 30% suspension). Mice were given a booster 1 week later, killed 3 days thereafter and spleen cells prepared, fused with the fusing line SP2/0-Ag14 (ref. 29) and hybridomas selected in hypoxanthine-aminopterin-thymidine medium (ref. 34 and S. Settle and J. Brown, personal communication). To analyse cytoplasmic proteins, hybridoma cells were washed twice in phosphate-buffered saline, PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2), pelleted, resuspended in 20 μ l PBS and lysed with 100 μ l of Nonidet P-40 (NP40) lysis buffer (0.5% NP40, 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 50 μ M phenylmethylsulphonyl fluoride). After 30 min on ice, nuclei and debris were pelleted. 15 μ l of hybridoma supernatant and 5 μ l of cytoplasmic protein were reduced with 15 μ l isoelectric focusing (IEF) sample buffer (8 M urea, 1.5 M 2-mercaptoethanol, 0.1 M potassium phosphate, pH 8.0) and loaded onto a horizontal polyacrylamide isoelectric focusing gel (5% acrylamide, 4 M urea, 1.6% ampholines, pH 3.5–10, 0.4% ampholines pH 4–6, 1% Triton X-100, 0.014% ammonium persulphate, 0.1% N, N, N', N'-tetramethylethylenediamine (TEMED)) and focused to equilibrium on an LKB Multiphor apparatus. After focusing, the proteins were electroblotted onto nitrocellulose in 0.7% acetic acid. Protein blots were blocked with 1% bovine serum albumin, 1% gelatin, probed with a rabbit anti-mouse C_{κ} antiserum, followed by a goat anti-rabbit horseradish peroxidase conjugate and developed with HRP Colour Development Reagent and H_2O_2 according to a procedure from BioRad.

are analysed without reduction of S-S bonds, we find that in both secreted and cytoplasmic proteins of transgenic κ secretors, the transgenic κ chain is associated with a heavy chain (μ or γ) (Fig. 2, lanes 1–4). All hybridomas of this type tested secrete the transgenic κ chain in association with heavy chain and do not secrete free light chains. Endogenous κ secretors that also have cytoplasmic transgenic κ chains do not have associated heavy chains (Fig. 2, lanes 5–8). We conclude that the endogenous κ chains are secreted as free light chains, whereas the transgenic κ chain alone is retained in the cytoplasm. These secretors of free endogenous κ have heavy-chain mRNA (Table 1) but a protein blot of reduced secreted and cytoplasmic protein probed with rabbit anti-mouse μ -, γ - or α -chain failed to detect any heavy chains in any hybridomas of this type (data not shown). Endogenous κ secretors that have no cytoplasmic transgenic κ chains secrete their endogenous κ chains in association with μ heavy chain (Fig. 2, lanes 9–12). Two non-secreting

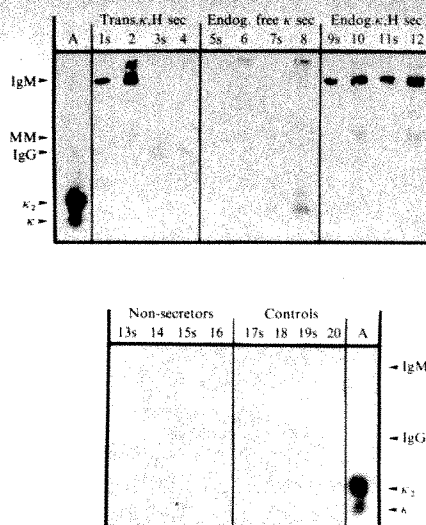


Fig. 2 Protein blot of unreduced hybridoma supernatants and cytoplasmic proteins. Each box of four lanes contains secreted and cytoplasmic proteins from two hybridomas of a particular type. Even-numbered lanes are cytoplasmic proteins; odd-numbered lanes are supernatants. Lane A, P-3 supernatant plus MOPC-41 free light chain (Litton Bionetics). Lanes 1–4, two representative transgenic κ secretors; lanes 5–8, two endogenous free light-chain secretors having cytoplasmic transgenic κ chains; lanes 9–12, two endogenous κ secretors having no cytoplasmic transgenic κ chains; lanes 13–16, two non-secretors (the amount of transgenic κ in the cytoplasm is insufficient to be detected in this gel system, probably because the light chains migrate over a wide range, and are not concentrated into a tight band as in IEF gels); lanes 17, 18, a hybridoma that has lost the transgenic κ gene; lanes 19, 20, fusing line SP2/0. MM, IgM monomer; κ_2 , κ -chain dimer; κ , κ monomer.

Methods: Supernatants and cytoplasmic proteins were prepared as described in Fig. 1 legend. 20 μ l of supernatant and 15 μ l of cytoplasmic proteins were run unreduced on a 1% agarose, 2.5% acrylamide gel (containing 0.2 M potassium phosphate, pH 7.2, 0.1% SDS, 0.5 M urea, with a running buffer of 0.075 M sodium potassium phosphate, pH 7.2, 0.1% SDS and 0.5 M urea). The proteins were electroblotted in 25 mM Tris base, 192 mM glycine, 20% methanol towards the anode, probed and developed as in Fig. 1. The rabbit anti-mouse κ antiserum does not cross-react with any other heavy or light chains (data not shown).

hybridomas do not produce heavy chains (Fig. 2, lanes 13–16). No κ or heavy-chain protein is observed in a hybridoma that has lost the transgenic gene (Fig. 2, lanes 17, 18) and no κ or heavy chain is detected in the fusing line (Fig. 2, lanes 19, 20).

Hybridoma transcription

In order to determine which κ genes are transcribed, we hybridized total RNA from each hybridoma on Northern blots with cDNA probes for κ , λ and three C_H genes (μ , $\gamma 2b$ and α) (data not shown). The fusing line SP2/0 contributes C_{κ} transcripts, a V_{κ} M.21 homologous transcript and a $\lambda 1$ transcript; transcripts from endogenous light-chain genes therefore cannot be distinguished. However, the fusing line did not produce μ or γ mRNA. All hybridomas produced at least one type of C_H -containing transcript (Table 1). In those hybridomas which produce heavy chains, the heavy-chain transcripts are consistent with the protein detected. Those hybridomas that have no heavy-chain proteins and secrete free endogenous κ chain do have full-sized μ or γ mRNA. Probably, these heavy-chain mRNAs are derived from faulty heavy-chain gene rearrangements and are apparently not translated into recognizable heavy chains.

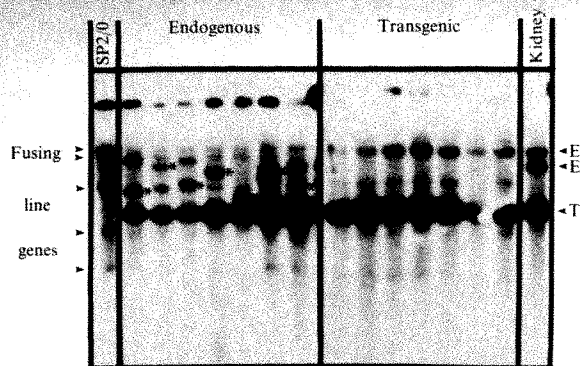


Fig. 3 Southern blot analysis of DNA from transgenic hybridomas. Left, DNA from seven different endogenous κ secretors; right, from seven different transgenic κ secretors. The SP2/0 lane indicates C_{κ} genes contributed by the fusing line, not all of which are maintained in the hybridomas. Kidney DNA from a heterozygous transgenic mouse indicates bands from the *SJL* allele (lower E), *C57BL/6* allele (upper E) and the integrated κ gene (T). The hybridomas shown either were derived from a spleen cell donor homozygous for the *C57BL/6* allele, or have lost the chromosome bearing the *SJL* κ gene. All transgenic DNAs have the 5.2-kilobase *Bam* fragment from the functionally rearranged transgenic κ gene. Note that this fragment is absent from SP2/0.

* Endogenous rearranged κ genes. DNA was prepared from hybridoma cell lines as described previously⁵, digested with restriction endonuclease *Bam*HI, electrophoresed at 20 μ g DNA per lane in a 0.9% agarose gel, blotted onto nitrocellulose and probed with C_{κ} sequence (M13-CK)¹⁸.

Characterization of hybridoma light-chain genes

It was important to determine directly whether the microinjected κ gene had an effect on endogenous immunoglobulin gene rearrangement. DNA from all hybridomas was digested with several restriction endonucleases and analysed on Southern blots. We found rearranged endogenous C_{κ} genes among the endogenous κ secretors (Fig. 3). Among the transgenic κ chain secreting hybridomas, we have detected no endogenous κ gene rearrangements on testing with *Bam*HI (Fig. 3), *Eco*RI and *Pvu*II (not shown) restriction enzymes. All DNAs were also restricted with the enzymes *Hind*III and *Sac*I and probed with a DNA sequence corresponding to the 5' half of the J_{κ} - C_{κ} intron (data not shown). Again, no rearrangement of endogenous κ genes was seen in transgenic secretors, whereas hybridomas producing endogenous κ chains did show κ gene rearrangements. No rearrangement of endogenous λ light genes in any hybridomas have been detected using Southern blot analysis of λ light-chain genes with restriction endonucleases *Bam*HI and *Xba*I, and using a V_{λ} probe (data not shown). Finally, Southern blots probed with a J_H probe indicate that the different transgenic

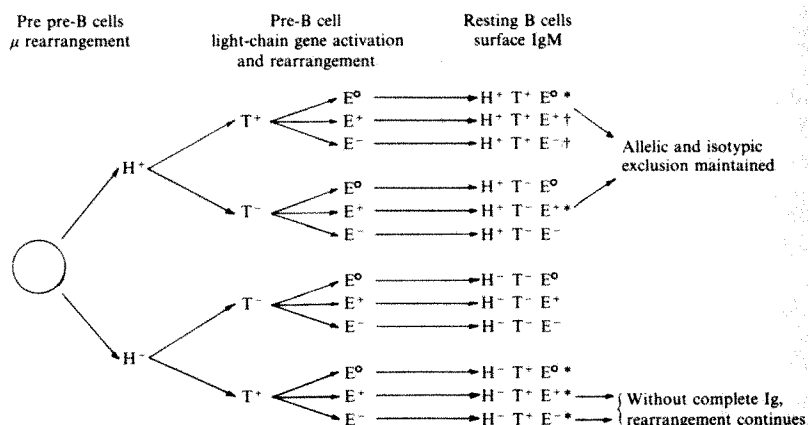
κ secretors have rearranged different VDJ_H genes (data not shown).

The analysis of the various types of hybridomas (Table 1) can now be summarized: in transgenic κ secretors, heavy chains are made which then associate with transgenic κ chains and the complete immunoglobulin molecule is secreted; no endogenous light chains are detected and no endogenous light chain genes are rearranged. In endogenous κ secretors that secrete free light chain, no heavy chains are made, the transgenic κ chain is present in the cytoplasm but not secreted and endogenous κ genes are rearranged. In endogenous κ secretors that secrete whole immunoglobulin, heavy-chain proteins are made, the transgenic gene is present but not expressed into protein and endogenous κ genes are rearranged, expressed and assembled into a complete immunoglobulin molecule. In non-secreting hybridomas, heavy chains have not been detected, the transgenic κ is present in the cytoplasm but not secreted, no endogenous κ chains are found in the cytoplasm but endogenous κ genes are present in either a germ-line or rearranged context. These data are consistent with the hypothesis¹⁴⁻¹⁶ that a complete, functional immunoglobulin (not just a functional light chain) has a role in turning off rearrangement. Cell types that have not been detected are first, transgenic κ secretors with cytoplasmic endogenous κ chains, suggesting that in cells where the microinjected gene is completely functional, endogenous κ chains are not produced; second, hybridomas that secrete both transgenic and endogenous κ chains, that is, allelic exclusion is observed; and third, hybridomas that secrete λ light chains. It is known that κ transgenic mice have normal amounts of λ mRNA in their spleen and λ chains in their serum (S. Ogden, K.A.R. and U.S., unpublished observations), but a λ -secreting hybridoma has not been found, perhaps because too few hybridomas have been characterized.

Conclusions

The isolation of B-cell hybridomas that express the microinjected κ gene described here confirms previous findings that the gene is expressed in B cells^{18,19}. We assume that the hybridomas represent a random sampling of B cells present in the transgenic mice; particularly that the transgenic κ secretors represent mature B cells and not pre-B cells immortalized by cell fusion before they had rearranged endogenous κ genes. First, preliminary data from Abelson virus-transformed, transgenic bone marrow cells demonstrate that pre-B cells exist which have μ mRNA but no transgenic κ transcripts (O. Witte, K. Dennis, J. St Claire, R.L.B. and U.S., unpublished results). Second, endogenous κ genes rearrange in hybridomas having no heavy chain but two different κ chains, whereas transgenic κ secretors have neither endogenous κ gene rearrangements nor cytoplasmic endogenous κ chains. Third, several of the transgenic κ secretors produce γ mRNA and protein, which indicates

Fig. 4 Various types of transgenic hybridomas within the context of normal B-cell development. Functionality is defined by the ability to be part of a complete immunoglobulin molecule. H, Heavy chain; T, transgenic κ chain. The T⁻ symbol does not mean that the transgenic κ genes rearrange, but represents the chance occurrence that a particular B cell inactivates the transgenic κ gene by some event. E, Endogenous light-chain genes. During the normal programme of B-cell development, a transgenic B cell undergoes three major events: rearrange a heavy-chain gene; activate or, by chance, inactivate the transgenic gene; and finally, rearrange (or not rearrange) endogenous light-chain genes. Options at each of these events produce the various types of B cells on the right. Allelic and isotypic exclusion is the result of forming a complete antibody molecule. °, Unrearranged; +, functionally rearranged; -, non-functionally rearranged gene (nomenclature of ref. 24). *, Cell types found; †, cell types not found, that is, rearrangement stops.



mature B cells. Finally, the normal distribution of mature B and T cells in transgenic spleens¹⁹ and the normal histology of spleen and lymph nodes (K. Gollahan and U.S., unpublished observations) reveals no gross abnormalities in lymphocyte development.

We do not know exactly when the transgenic κ gene becomes transcriptionally active relative to normal immunoglobulin gene expression (Fig. 4). The findings with transgenic Abelson pre-B-cell lines (see above) and with normal pre-B-cell hybridomas²³ suggest that μ rearrangement and transcription precedes κ transcription in immature B cells. In mature B cells, unrearranged light-chain genes are known to be transcribed^{23,24}, undermethylated²⁵ and DNase-I sensitive²⁶. If the transgenic κ chain is to have an effect, κ transcription must precede rearrangement. Transgenic κ gene transcriptional activation is shown to occur when normal germ-line κ genes are presumably activated—after μ rearrangement and before endogenous κ gene rearrangement in the scheme in Fig. 4. The isolation of $H^+T^+E^-$ cells (H, heavy chain gene; T, transgenic κ chain gene; E, endogenous light chain gene) but the lack of $H^+T^+E^+$ and $H^+T^-E^-$ cell types indicates that when a functional heavy chain is provided, the transgenic κ gene prevents endogenous light-chain gene rearrangement. The presence of $H^+T^-E^+$ type cells shows that when the transgenic gene is inactivated by chance, endogenous genes can indeed rearrange. Finally, $H^+T^+E^+$ and $H^+T^-E^-$ cell types show that without a heavy chain, the presence of the functional transgenic κ chain alone is insufficient to prevent endogenous immunoglobulin gene rearrangement. The results strongly support the hypothesis¹⁴⁻¹⁶ that the presence of a complete immunoglobulin molecule turns off rearrangement and thus establishes allelic exclusion of immunoglobulin genes. Our data do not address the regulation of λ or heavy-chain gene rearrangement. It seems likely, however, that λ - and heavy-chain gene rearrangements also stop when a complete immunoglobulin is made.

As the history of the hybridomas, or of the B-cells from which they are derived, is unknown, it is possible that the $H^+T^+E^+$ (E^-) cells were derived from H^+ cells. In that case a cell which originally was $H^+T^+E^-$ would have reinitiated rearrangement of endogenous κ genes after the loss of the H^+ chain. This cannot at present be ruled out. It seems likely, however, that the rearrangement machinery becomes permanently inactivated by differentiation into a B cell after the appearance of a complete immunoglobulin molecule. Defining the molecular basis of the feedback inhibition of immunoglobulin gene rearrangement will probably answer this question. If indeed the shutoff of rearrangement is permanent (and if T^+ is a functional protein) then the existence of $H^+T^+E^+$ (E^-) cells suggests that they have never been H^+ and consequently that the presence of heavy-chain protein may not be a prerequisite for light-chain gene rearrangement.

In considering the kind of heavy- and light-chain pairs which have the potential to stop further immunoglobulin gene rearrangement, a distinction must be made between preferential and random association. Clearly, specific light- and heavy-chain combinations are seen in immune responses to antigens like phosphorylcholine. This kind of preferential association, however, is a function of the ability of a pair of heavy and light chains to bind antigen, not of the intrinsic ability of the heavy and light chains to assemble. Several lines of evidence support random pairing of heavy and light chains^{22,27-29}. Preferential association of homologous heavy and light chains has been demonstrated only in competitive situations³⁰⁻³³. Discovery of two full-size κ chains in the myeloma S107 and the association of only one of those with heavy chain indicates that preferential association may have a role in allelic exclusion^{15,16}. In S107, however, the non-secreted κ chain has a deletion of two amino acids including residue 96 (refs 15, 16), which has been found to be critical for association with heavy chains²⁸. As postulated^{15,16}, myeloma S107 does not exemplify preferential association, but shows that a κ rearrangement and polypeptide can be nonfunctional in its inability to associate with heavy chain. Apparently, any heavy-light-chain association can shut off immunoglobulin gene rearrangement, as all heavy chains of transgenic secretors are different (data not shown) and we have found no case of a $H^+T^+E^+$ hybridoma.

Although no myelomas have been described which produce more than one functional κ chain, theoretically such cells might exist. First, it is conceivable that two κ alleles may functionally rearrange and become transcribed at exactly the same time. Second, it is also possible that a particular κ gene, although rearranged, could be transcribed at too low a rate to produce enough κ protein to achieve a threshold level of the heavy-light chain complex required for feedback.

Analysis of these hybridomas provides the first direct evidence for the hypothesis¹⁴⁻¹⁶ that a complete immunoglobulin molecule turns off the process of rearrangement. The mechanism for this shutoff is now being investigated. Our results support the idea that any light chain can associate with any heavy chain, and confirm that the significant mechanism for allelic exclusion is the feedback-mediated shutdown of rearrangement.

We thank S. Settle for help with hybridoma production; B. Arp for production of probes, breeding of mice and help with the figures; R. O'Brien for determination of positive transgenic mice; J. Clagett for the loan of focusing equipment; and J. Brown, R. Palmiter, T. Stanton and C. Sibley for advice. We also thank R. Near, J. Miller, R. Perry and G. Sonenshein for DNA probes, and R. Finical and C. Ritchie for typing the manuscript. This research was supported by NIH grants CA/AI 25754, CA 35979, DE 02600 and HD 17321 and NSF grant PCM 81-07172. K.A.R. was supported by NIH-NIGMS NRSA no. GM-07266.

Received 9 August; accepted 25 September 1984.

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Sequence and expression of transcripts of the human T-cell receptor β -chain genes

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T lymphocytes can produce mRNAs from the first or second constant region of the T-cell antigen receptor β -chain. A 1.3-kilobase (kb) transcript encoding the mature protein contains V, J and one of the two C region sequences, but does not always contain D sequences. A 1.0-kb mRNA, found mainly in immature T cells, does not contain V sequences and can result from transcription that initiates upstream from an unrearranged J element.

B AND T lymphocytes identify and eliminate a large variety of foreign antigens. Understanding the mechanisms of antigen recognition and subsequent activation of these cells is central to our investigation of the immune response. One clear difference between antigen recognition by B and T lymphocytes is that the former can bind antigens by themselves, whereas T cells recognize foreign antigens in conjunction with the major histocompatibility complex (MHC) gene products, a phenomenon known as MHC restriction^{1,2}. Although the immunoglobulin proteins and genes involved in the recognition of antigens by B cells are well characterized^{3,4}, the T-cell antigen receptor, responsible for the binding of antigens by T cells, has only lately been amenable to study.

Human⁵ and murine⁶ T-cell-specific cDNA clones that specify the β -chain⁷ of the T-cell antigen receptor have been isolated. The genes are similar in organization to the immunoglobulin genes with separate variable (V), diversity (D), joining (J) and constant (C) region segments⁸⁻¹¹, rearranging in T cells^{12,13} to produce immunoglobulin-like proteins^{5,6}. There are two closely linked groups of J and C regions in the murine germ-line genome, arranged 5' $J_{\beta 1}$ - $C_{\beta 1}$ - $J_{\beta 2}$ - $C_{\beta 2}$ 3' (refs 10, 11). A similar organization is found in the human genome (B. Toyonaga, personal communication). Each J region is a cluster of independent J segments⁸⁻¹¹ with a closely associated D element on the 5' side¹⁴⁻¹⁶. The distance from the germ-line V genes to the J-C region is unknown¹⁷. We seek here to characterize the transcription products of the rearranged genes of T-cell lines and populations of T cells.

Two constant regions in human β -chain

Analysis of β -chain protein and DNA rearrangement data suggest that there is variability between different T-cell receptor β -chains. To characterize the different transcripts produced from the β -chain region, we constructed cDNA libraries from polyadenylated RNA extracted from the T-cell lines Jurkat or HPB-ALL. Jurkat was of particular interest because of the existence of monoclonal antibodies which recognize both its α - and β -chains (A. Weiss and J. Stobo, personal communication), and because genomic Southern blots show the same pattern of rearrangement as the T-cell line MOLT-3 (ref. 12), from which the original β -chain cDNA clone YT35 was derived⁵. Twelve cDNA clones that hybridized to YT35 were isolated from the Jurkat library and their restriction enzyme sites mapped. These clones fall into two groups: seven that are indistinguishable from YT35, such as JUR- $\beta 1$, and two which are slightly different (Fig. 1). When compared with JUR- $\beta 1$ and YT35, JUR- $\beta 2$ has an extra BglII site and lacks an EcoRV site. In addition, it is only 0.85 kilobases (kb) long, compared with the full-length size of 1.3 kb for JUR- $\beta 1$.

Nucleotide sequencing of JUR- $\beta 1$ and JUR- $\beta 2$ clones demonstrates that JUR- $\beta 1$ is identical to YT35 (data not shown). This surprising finding indicates that these two different T-cell

lines use the same V, D, J and C region sequences, and explains why Southern analysis shows the same pattern of rearrangement. The sequences of JUR- $\beta 1$ and JUR- $\beta 2$, together with the protein sequence deduced from their only long open reading frame, are compared directly in Fig. 2. JUR- $\beta 2$ extends only a few nucleotides 5' of J. There is some homology between the J segments of the two clones and almost perfect homology in the C protein-coding region. The JUR- $\beta 2$ J segment has a very similar nucleotide sequence to the murine $J_{\beta 2.3}$ element (Fig. 2). In the C region, there are 17 nucleotide differences, only six of which lead to amino acid substitutions. Perhaps the most significant is the transversion at position 992 in JUR- $\beta 1$ that converts the termination codon TGA to a serine codon AGA, producing a protein two amino acids longer. Although the differences between these two C region sequences may reflect polymorphisms between two human chromosomes, it is more likely that they represent transcripts from different pre-existing constant regions in the genome because the 3'-untranslated sequences show no similarities. These two Jurkat transcripts probably correspond to the two distinct but highly homologous C-region segments about 7 kb apart in the human germ-line DNA (B. Toyonaga *et al.*, in preparation). JUR- $\beta 1$ and YT35 are transcribed from the 5' $C_{\beta 1}$ whereas JUR- $\beta 2$ is transcribed $C_{\beta 2}$.

Polyadenylation in JUR- $\beta 2$ occurs at a guanosine residue 20 bases from the polyadenylation signal (marked in Fig. 2). Polyadenylation of the murine β -chain mRNA also occurs at guanosine residues¹¹.

Optional D segments

The T-cell line, HPB-ALL¹⁸, shows a different pattern of rearrangement from MOLT-3 and Jurkat (unpublished data). Comparison of the sequence of a YT35 hybridizing clone, HPB- $\beta 2$, from the HPB-ALL cDNA library¹⁹ with that of JUR- $\beta 2$ indicates that the former is transcribed from the $C_{\beta 2}$ gene, but there are two silent mutations in the C region and a frameshift in the 3'-untranslated sequences (these positions are marked in Fig. 3). We suggest that these changes result from polymorphisms in the human population. A comparison of the nucleotide and deduced protein sequences of HPB- $\beta 2$ with JUR- $\beta 1$ is shown in Fig. 3. The homology between the C coding regions is very high, but there is only random homology in the untranslated region, as is the case between the $C_{\beta 1}$ and $C_{\beta 2}$ regions of JUR- $\beta 1$ and JUR- $\beta 2$. The homology in the J segment (45% overall) is strongest in the middle, which is the most highly conserved region in the murine J segments at both the nucleotide and amino acid level. The J region has 74% identity (35/47) with the murine $J_{\beta 2.5}$ element¹⁰. The V regions are different, but clearly related. The regions of highest homology are clustered around the 3' end, where there is a cysteine residue in the protein sequence, and around position 305 in JUR- $\beta 1$. To align the V regions properly, a gap of 15 nucleotides (five codons) must be introduced into HPB- $\beta 2$ between the J and V sequences; this

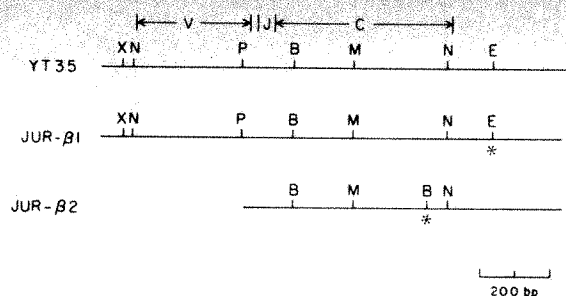


Fig. 1 Restriction enzyme maps of two representative cDNA clones of the T-cell receptor β -chain from the T-cell line Jurkat. Polyadenylated RNA was selected, double-stranded cDNA was generated, tailed with deoxycytidine and inserted into the deoxyguanosine-tailed *Pst*I site of pBR322, as described previously⁵. Colonies that contained cDNA clones to the β -chain were selected by filter hybridization to a nick-translated YT35 probe. The clones were mapped with *Xba*I (X), *Nco*I (N), *Pvu*II (P), *Bgl*II (B), *Msp*II (M) and *Eco*RV (E) using standard techniques. The restriction enzyme map of YT35 is included for comparison. Asterisks mark the restriction sites that differ between JUR- β 1 and JUR- β 2.

gap corresponds exactly with the *D* segment from JUR- β 1 and YT35. It seems that the HPB-ALL mRNA does not include a *D* element.

mRNA expression

To determine the relative frequency of expression of the mRNA from the two *C* gene segments and whether mRNAs from both are transcribed often in a single cell (as we found with Jurkat by sequencing cDNA clones), we performed Northern gel analysis. RNAs from thymocytes, peripheral blood T cells and the T-cell lines Jurkat, MOLT-3 and CEM were hybridized with *V* and *C* coding-region probes and untranslated 3'-region probes of *C* _{β 1} and *C* _{β 2} (Fig. 4). As expected, none of the probes hybridized to B-cell RNA. Figure 4a shows that two bands can be detected in T cells with the constant-region probe—a strong one at 1.3 kb and a weaker one at 1.0 kb. In mature peripheral T lymphocytes, there is only about 10–20% as much expression of the β -chain as in immature thymocytes. The 1.0-kb transcript does not hybridize to the *V* probe (Fig. 4b), so only the 1.3 kb transcript containing *V*-region sequences corresponds to the mature mRNA comprising *V*, (*D*), *J* and *C* segments. Peripheral T lymphocytes and the leukaemic T-cell line CEM do not have this 1.0-kb mRNA (Fig. 4a, c, d). Hybridization of the *V*-region probe to the 1.3-kb band (Fig. 4b) is strong for MOLT-3 and Jurkat RNA, which we expected, as they are identical to the probe. The CEM RNA hybridizes only slightly, indicating that there is limited homology between *V* _{β M3.2} (from YT35) and *V* _{β CEM1}, as there is between *V* _{β M3.2} and *V* _{β HPB1} (from HPB- β 2; Fig. 3). Thymocytes and peripheral T cells are a heterogeneous mixture of cells having different antigen specificities; thus each expresses a different *V* region and hybridization of their RNAs to the YT35 *V*-region probe is weak.

The 3' probes from the untranslated regions allow us to distinguish transcription from the two *C* _{β} -region genes (Fig. 3c, d). Both the 1.3- and 1.0-kb mRNAs from MOLT-3 are transcribed from the *C* _{β 1} region. In contrast, the 1.3-kb mRNA from Jurkat is transcribed from *C* _{β 1} while the 1.0-kb message is transcribed from *C* _{β 2}, and the 1.3-kb mRNA from CEM is transcribed from *C* _{β 2}. Of the cell lines tested, only Jurkat transcribes from both *C*-region genes. Surprisingly, thymocytes show a preponderance of transcription of *C* _{β 2} for both sizes of transcripts, but the distribution in the mature peripheral T cells is nearly equal.

JUR- β 2 sequences

The Jurkat 1.3-kb transcript is from *C* _{β 1} and the 1.0-kb transcript from *C* _{β 2} (Fig. 4). Apparently, JUR- β 2, which is transcribed from *C* _{β 2}, is a cDNA clone of the 1.0-kb transcript. Figure 2 compares the *J* region from JUR- β 2 with the murine germ-line *J* _{β 2.3} segment. Within the coding region of *J* _{β 2.3} there is 80%

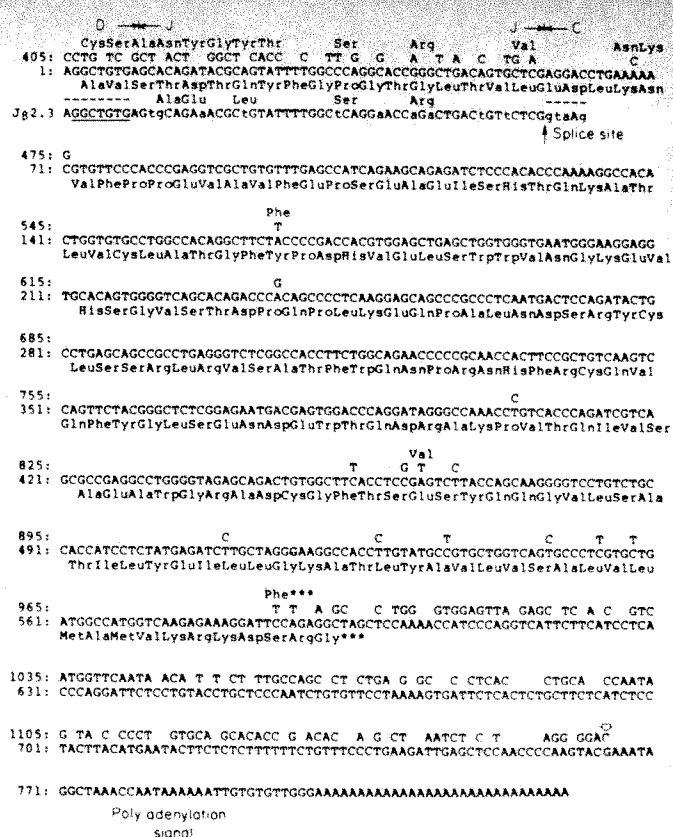


Fig. 2 The nucleotide and deduced amino acid sequence of clone JUR- β 2, and comparison with JUR- β 1. The DNA sequence was determined using the dideoxy chain-termination method²⁸. The insert was released from the pBR322 vector, isolated by preparative agarose gel electrophoresis, then digested with one of several four-cutter restriction enzymes and cloned into the single-stranded phage vector M13mp9. The sequence of JUR- β 2 is shown in full with its deduced protein sequence beneath; the sequence above JUR- β 2 indicates positions at which JUR- β 1 is different. The open arrows indicate the 3' limit of our sequence of JUR- β 1. Diversity (*D*), joining (*J*) and constant (*C*) regions, as determined for YT35 (ref. 8) (identical to JUR- β 1), are shown. The polyadenylation signal of JUR- β 2 is underlined. Also shown is a comparison of the *J* region of JUR- β 2 with the murine *J* _{β 2.3} element¹⁰: the nucleotides that are the same as those in JUR- β 2 are given in upper case, differences in lower case letters. The deduced amino acid sequence of *J* _{β 2.3} is shown above the nucleotide sequence only where it differs from JUR- β 2. The untranslated regions are indicated by dashes; the splice site is marked by an arrow and the heptamer recombination recognition sequence is underlined.

identity (39/49) to JUR- β 2. This homology ends abruptly at the 3' end, as expected, at the splice site. At the 5' end, however, the homology continues past the *J* _{β 2.3} coding region into the heptamer of the recombination recognition signal. It seems, therefore, that the 1.0-kb transcript from Jurkat initiates upstream from an unrearranged *J* element.

Discussion

Our analysis of the structure and expression of cDNA clones has revealed much about the rearrangement and transcription of the β -chain and about the structure of the protein. We have shown directly, by DNA sequencing, that at least two human *V*-gene segments can form mature β -chains of the T-cell receptor. These *V*-gene segments, although quite different, are clearly related, particularly around the cysteine residue near the 3' end that is also conserved in immunoglobulin proteins²⁰. We have found that at least three *J* segments in the human are active, and that both constant-region genes, *C* _{β 1} and *C* _{β 2}, can be incorporated into the mRNAs encoding functional proteins. Each of these regions has some similarity in protein sequence to the analogous immunoglobulin region and there is a similar placement of introns: approximately between the signal peptide and

AspSerGlyMetProGlu LysMetProAsnAlaSerPhe Thr LysIleGln
 281: TTCA GG TGCC GAG AAGAT CCTAA GCA CATT CACT GA CC
 1: GAAGAGGAAGCTTCCTGATCGATTCTCAGCTCGCCAGTTCCTTAAGCTCTGAGCTGAATGTGA
 GUGUGUGSerPheProAspArgPheSerAlaArgGlnPheProAsnTyrSerSerGluLeuAsnValAsn

 ProSerGluProArg Val Phe PheSerThrCysSerAlaAsnTyr
 351: GC CAGAA CCA A TG CT T TCTCGACCTGTTCGG TAACT
 71: ACCGCTGTGTGGGGGACGCGCCCTGTATCTCTGCGACGACGCT CCGGGAA
 AlaLeuLeuGlyAspSerAlaLeuTyrLeuCysAlaSerS erArgLys

 GlyTyrThr Ser Thr Val AsnLys
 421: TGG T CAC TT G G CA T AACC TG A C G
 126: AACCCATTACTTCGGGCGAGCAGCGGCTCTGGTCTGAGGACCTGAAACCGTGTCCCAACCGAG
 ThrHisTyrPheGlyProGlyThrArgLeuLeuValLeuGluAspLeuLysAsnValPheProProGlu

 491: GTGCTGTGTGGGACCATCAGAGCAGAGATCTCCCAACCCAA/AGGCCACACTGGTGTGCTGCGGCA
 196: ValAlaValPheGluProSerGluAlaGluIleSerHisThrGlnLysAlaThrLeuValCysLeuAlaThr

 Phe
 561: T
 266: CAGGCTTTCACCCGACCCAGCTGGAGCTGAGCTGGTGGTGAATGGGAAGGAGGTGCACAGTGGGTCAG
 GlyPheTyrProAspHisValGluLeuSerTrpValAsnGlyLysGluValHisSerGlyValSer

 631: CACAGACCCGACGCCCTCAAGGAGCAGCCGCCCTCAATGACTCCAGACTACTGCTGAGCAGCCGCTG
 336: ThrAspProGlnProLeuLysGluGlnProAlaLeuAsnAspSerArgTyrCysLeuSerSerArgLeu

 701: AGGGTCTCGGCCACCTTCTGGCAGAACCCGCAACCACTTCGGCTGTCAAGTCAGTCTTACGGGCTCT
 406: ArgValSerAlaThrPheTrpGlnAsnProArgAsnHisPheArgCysGlnValGlnPheTyrGlyLeuSer

 771: C
 476: CGGAGATGACGAGTGGACCCAGGATAGGGCCAAACCTGTCAACGAGATGCTCAGCGCCGAGGCTGGG
 GlnAsnAspGluTrpThrGlnAspArgAlaLysProValThrGlnIleValSerAlaGluAlaTrpGly

 Val
 841: T G T C C
 546: TAGAGCAGACTGTGCTTCACTCCGAGTCTTACCAGCAAGGGGTGCTGTCTGCCACCATCTCTATGAG
 ArgAlaAspCysGlyPheThrSerGluSerTyrGlnGlnGlyValLeuSerAlaThrIleLeuTyrGlu

 C C C T T T
 911: ATCTTCTAGGGAAGGCCACCTTGTATGCCGTGCTGCTCAGTGGCCCTGCTGATGGCCATGGTCAAGA
 616: IleLeuLeuGlyLysAlaThrLeuTyrAlaValLeuValSerAlaLeuValLeuMetAlaMetValLysArg

 Phe***
 981: T T A GC C TGG GTGGATTA GAGC TC A C GTCATGTTCAATA ACA
 686: GAAAGGATTCAGAGGCTAGCTCCAAACCATCCAGGTCTTCTTCATCTCACCAGGATTCCTCTGT
 LysAspSerArgGly***

 1051: T T CT TTGGC G GC TC GAAG GGTGTC A CTCTGTGCATC CAA AGATA CC CCT TTGT
 756: ACCGTGCTCCAGATCTGTGTTCTTAAAGGATTTCTACTCTGCTTCTATCTCTCTACTTACATGAATAC

 1121: CATG A ACC GCACAC CA GCTGA A CTC CTA C AG
 826: TTCTCTCTTTTTTCTGTCTCCCTGAAGATTGAGCTCAACCCC : 868

Fig. 3 The nucleotide and deduced amino acid sequence of clone HPB- β 2 from the T-cell line HPB-ALL, and comparison with JUR- β 1. The cDNA was cloned¹⁹ and selected as described in Fig. 1 legend. Sequencing was done partly as described in Fig. 2 legend and partly using the Maxam and Gilbert procedure²⁹. The sequence of HPB- β 2 is shown in full with its deduced amino acid sequence beneath. Positions at which JUR- β 1 differs from HPB- β 2 are presented above the sequence of HPB- β 2. Gaps introduced to maintain the alignment are indicated by dots. V, variable; D, diversity; J, joining; and C, constant regions. Differences between JUR- β 2 and HPB- β 2 in the constant and 3' untranslated regions are underlined.

the mature protein in the V gene¹⁵ and between the J and C regions⁹⁻¹¹. Such a resemblance points to the similarity of function of the immunoglobulin and T-cell receptor proteins and their common evolutionary origin.

There are intriguing differences, however, between these families of proteins. The immunoglobulin light chains are closest in size to the β -chain, but the C-gene segment is composed of four exons⁹⁻¹¹ and has a transmembrane segment^{5,13} like the immunoglobulin heavy chains. Also in common with the heavy chain, the β -chain uses a D segment^{8,9,14-16}. Analysis of the recombination recognition signals adjacent to the V, D and J germ-line segments^{8,9,14-16} reveals striking similarity to those of immunoglobulins^{21,22}.

These signals are composed of a palindromic heptamer sequence proximal to the coding region of the element, and a distal thymidine-rich nanomer separated from the heptamer by a non-conserved spacer of either 12 or 23 nucleotides. The size of the spacer is critical because one element can rearrange next to another only if one has a spacer of 12 bases and the other is of 23 bases^{21,22} (termed the '12/23' rule, or the 'one turn/two turn' rule, because of the number of helical turns separating the signals). The V and J elements of the immunoglobulin heavy-chain genes have a 23-base spacer so that they can each only rearrange to a D_H segment, where the spacer is 12 bases long on both sides. In contrast, the β -chain V segment has a 23-base spacer, the J _{β} segments have 12-base spacers, and the D _{β} elements have 12-base and 23-base spacers respectively on the 5' and 3' side. Thus, if the size of the spacer is the only factor determining which elements can join, it is theoretically possible for a V _{β} gene to recombine with a J _{β} segment, omitting a D _{β} element.

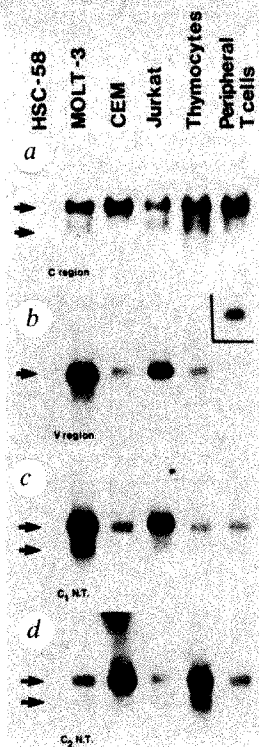


Fig. 4 Northern gel analysis of RNA from T cells using probes to the V region, C coding region and 3'-untranslated regions of C _{β 1} and C _{β 2}. *a*, The protein-coding C _{β 2} BglII fragment of JUR- β 2; *b*, a V _{β M3.2} PstI-AluI fragment of JUR- β 1; *c*, an AluI-PstI fragment of JUR- β 1 representing the 3'-untranslated sequences of C _{β 1}; *d*, an RsaI and RsaI-PstI fragment of JUR- β 2 representing the 3'-untranslated region of C _{β 2}. Arrows, positions of the 1.3-kb and 1.0-kb transcripts. The inset in *b* is a longer exposure of the 1.3-kb band from peripheral T-cell RNA. Unfractionated RNA from the human B-cell line HSC-58, from the human T-cell lines MOLT-3, CEM and Jurkat, from human thymocytes and from peripheral T cells was extracted and 10 μ g electrophoresed in each lane, except the peripheral T cells in *a* where 50 μ g were used, as described previously⁵. The probes were nick-translated after isolation from preparative agarose gels.

HPB- β 2 appears to be the result of such a recombination, lacking the precise sequence that corresponds to the D_{BYT35} segment of JUR- β 1 and YT35. There are three possible explanations for this: (1) there is no D element in the recombined gene and the V and J elements are similar in length between HPB- β 2 and JUR- β 1; (2) the V segment is shorter in HPB- β 2 (V_{HPB1}) than in JUR- β 1 (V_{BM3.2}) and the D element is homologous to the cysteine encoding 3' end of V_{BM3.2}, which is absent from V_{HPB1}; (3) the J element is shorter in HPB- β 2 than in JUR- β 1, the D segment making up the length, and is similar to the 5' end of a J segment. All the identified genomic J _{β} segments are of a similar length^{9,11-14}, so we believe that the simplest and most likely explanation is that there is no D segment in HPB- β 2. Although we suggest that the mechanism for this is direct recombination between the V_{HPB1} gene segment and the J _{β} element, we cannot exclude the possibility that the V_{HPB1} first recombined with a D _{β} element so that only the 3' recombination recognition sequences, with the 23-base spacer, remained subsequently rearranging to the J _{β} element.

Another consequence of unequal spacer sizes in the recombination signals that flank the D _{β} elements is that some β -chains may include two or more D segments via direct D-D joining. The presence or absence of D _{β} segment(s) may be a mechanism for increasing β -chain variability and thus expanding the T-cell receptor repertoire.

Our finding that the cell lines Jurkat and MOLT-3 produce identical mRNAs for the β -chain was unexpected. We have eliminated the possibility that these lines are the same by

histocompatibility locus antigen (HLA-A/B) typing (J. Falk, unpublished observations) and OKT surface marker typing²³; nor is it possible that the original cDNA clones were interchanged because we cloned YT35 into the *Bgl*III site⁵ of pFP502EB5 (ref. 24) whereas JUR- β 1 was cloned into the *Pst*I site of pBR322. The strong hybridization of the YT35 V-region to Jurkat RNA (Fig. 4b) provides independent evidence that YT35 and Jurkat use the same V_{β} -gene fragment. We do not know whether the production of this same mRNA in two transformed lines is related to tumorigenesis.

Preliminary examination of the β -chain rearrangements in bone marrow samples of 13 T-cell acute lymphoblastic leukaemia patients suggests a limited number of rearrangement patterns²⁵. This does not represent a limited repertoire of possible recombinations. There may be some preferred rearrangements in early thymocytes after they have become transformed, or some rearrangements may predispose the cells to transformation. This latter possibility is of particular interest in view of the hypothesis of AKR retroviral leukaemogenesis of Weissman and co-workers²⁶ which suggests that autostimulation of the T-cell receptor leads to a proliferative state during which the cell is more susceptible to transformation.

It has been shown that 1.0-kb mRNAs from thymocytes contain sequences from 5' of a D_{β} element rearranged to a J_{β} segment but not a V_{β} gene^{14,15}. In contrast, this transcript from the cell line Jurkat, represented by cDNA clone JUR- β 2, appears to contain sequences 5' to a J_{β} element rearranged neither to a D_{β} element nor to a V_{β} element. Unfortunately this clone does not extend further 5', but the limited sequence available is identical to the heptamer sequence 5' of the murine $J_{\beta 2.3}$, but different from $D_{\beta 1.1}$ (refs 14–16), $D_{\beta YT35}$ and the highly conserved 3' end of $V_{\beta M3.2}$ and $V_{\beta HBP1}$.

The chromosomal origin of this mRNA is unknown. The mechanism activating transcription of the functionally rearranged β -chain may induce also an aberrant transcript from the β -chain locus on the other chromosome which may have

D - J rearrangement; the activation may, however, be localized to one of the two chromosomes, including both $C_{\beta 1}$ and $C_{\beta 2}$. The question of whether the β -chain locus on both chromosomes can be simultaneously transcribed can be resolved with a cell line that uses $C_{\beta 1}$ for the 1.0-kb mRNA and $C_{\beta 2}$ for the 1.3-kb mRNA, where $C_{\beta 1}$ would be deleted.

Two major concerns of immunologists are the mechanisms of elimination of self-reactive T cells during T-cell ontogeny and of MHC restriction. Could the 1.0-kb mRNA be involved in these processes? The discrete and constant size of this transcript in thymocytes, Jurkat, MOLT-3 and other cells implies that there is a specific point for transcription initiation, unique to a particular cell type. Apparently, transcription initiation may occur upstream from an unrearranged J segment or from a J element recombined with a D segment. We do not know whether the smaller message is expressed simultaneously with the larger one in thymocytes as it is in MOLT-3 and Jurkat, which are transformed and thus may be abnormal, or whether pack cells have only one species of 1.0-kb mRNA. The T-cell-specific message clones by Tonegawa's group²⁷ also has a smaller size class. This may be a common property of this gene family.

An interesting difference between thymocytes and mature peripheral T cells is the preferential expression of $C_{\beta 2}$ in the thymocytes, in contrast to the situation in BALB/c mice, where $C_{\beta 1}$ is the most prevalent thymic transcript¹¹. We do not know whether this is due to species differences, or variability between individuals. Further characterization of β -chain expression should assist us in our attempts to understand the mechanisms by which a functional T-cell receptor protein is generated.

Y. Yoshikai, Y. Yanagi and S.P.C. are recipients of awards from the MRC of Canada. C.T. is a scholar of the Leukemia Society of America. P.V.de.E. is supported by a NATO science fellowship obtained through the Dutch Organization for the Advancement of Pure Research (ZWO). This work was funded by the MRC of Canada, the NCI of Canada, and NIH grants A115066 and A117651. We thank G. Siu for helpful comments.

Received 9 July; accepted 2 October 1984.

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LETTERS TO NATURE

Inflation in the universe and time asymmetry

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I previously argued that the origin of time asymmetry is to be sought in the processes that characterized the very early cosmological epochs, and that the recent inflationary scenario provides a crucial element in our understanding of this origin¹. Here, I develop this thesis further, in the light of criticisms by Page². I argue in particular that any of the "initial states" that appear to be permitted by quantum gravity, if succeeded by a period of inflation, would have wound up the universe to the state from which, as indicated by increasing entropy, it is now winding down.

The universe is ordered in two distinct ways. First, matter (for example stars) is generally found in a state rather far from thermodynamic equilibrium. Second, the universe is mysteriously regular on a larger scale, with only a moderate degree of structure, occasioned by the clumping of material into stars, galaxies, galactic clusters and so on. The cosmological gravitational field is therefore rather smooth. Both these forms of order are gradually degenerating, as matter is processed and loses free energy (as in stellar evolution for example) and as gravity causes the irreversible collapse of objects and systems towards, ultimately, a black hole state. This universal tendency may be considered as a one-way approach to thermodynamic equilibrium and maximum entropy in accordance with the second law of thermodynamics³. But the irreversible rise in cosmic entropy associated with this decay raises the question of how the universe achieved this order initially.

The central problem in addressing the above question is not in doubt. If the fundamental laws of physics are time-asym-

metric, then the observed time asymmetry of the world can only be explained by appealing to a special choice of initial state. (I ignore here the possibility of T-violating laws, as they are irrelevant to the issues under discussion.) Two questions then arise. First, can we identify the physical mechanisms whereby the special initial state evolves into the present (and projected future) state of the universe in a time-asymmetric fashion? And second, can we find reasons why the special initial state should be regarded as unremarkable, 'natural' or even inevitable? The first of these latter two questions is physical, the second essentially philosophical, and strongly model-dependent.

The first person to address questions 1 and 2 was Boltzmann, who treated a simple model gas. He was able to prove, in his famous H-theorem⁴ that, under the impact of intermolecular collisions, the entropy of the gas would rise with time whenever it found itself in a condition of 'molecular chaos'—that is, lacking correlations in the positions and motions of the molecules³. Applying an extrapolation of this theorem to the universe as a whole demands an answer to the second question. One might suppose that if the universe were initially in a state of 'cosmic molecular chaos', then subsequently the entropy would rise, during which phase the universe would behave time-asymmetrically. But now we come up against the question of how the universe achieved its state of 'molecular chaos' at less-than-maximum entropy in the first place.

One unscientific and metaphysical answer is simply to assume that the universe was made that way, that is, created in a special initial state. Another answer (the one suggested by Boltzmann) is that, in a universe which is infinite in time and/or space, and subject to time-symmetric laws, such a state is bound to occur purely by chance fluctuations (and occur infinitely often). The frequency of such a near-miracle is absurdly small, but—to use an anthropic explanation—because the existence of life depends on a source of free energy, we could not possibly find ourselves living in the much-more-probable state of thermodynamic equilibrium.

The above reasoning unfortunately contains a serious flaw. There seems to be no obvious reason why a fluctuation region should be of Hubble proportions in order that observers arise. Because the magnitude of fluctuations is exponentially related to their (im)probability, it would seem to be overwhelmingly more likely that a random observer would find himself in, say, a one-galaxy, or even a one-star, universe than in one populated by myriads of similar systems⁵. In short, the anthropic principle seems to suggest that we should observe a universe of minimal order consistent with the existence of observers.

It is against this general background of ideas that the inflationary universe scenario must be assessed. Briefly the scenario goes like this^{6,7}. At some early moment the universe finds itself in a quantum state known as a false vacuum, which is characterized by a huge energy density and negative pressure. The gravitational effect is identical to that of Einstein's cosmological constant, so that the universe embarks on a phase of exponential expansion (inflation). During this time the universe resembles a portion of de Sitter space. Eventually the inflationary phase ends, and the energy from the false vacuum is deposited into matter and radiation fields. At this stage, irregularities present initially will have been smoothed to insignificance by the enormous distension of space. Subsequently the universe evolves according to the standard hot big bang model.

In ref. 1 I argued that the process of inflation is responsible for 'winding up' the cosmic mechanism, by ensuring that the post-inflationary state is relatively smooth, hence enjoying a gravitational entropy much less than the maximum permissible. The apparent paradox that the universe has thereby progressed from a high to a low entropy state during the inflationary phase is resolved by noting that the universe is not a closed system (even if spatially finite) because free energy is not conserved in cosmology. On account of the negative pressure of the false vacuum, non-gravitational energy is actually created (per unit comoving volume) during inflation.

Boltzmann was faced with the question of how the universe achieved a state of (1) less than maximum entropy and (2) no correlations (molecular chaos). The situation here is directly analogous. We too have to explain how the universe arrived in the false vacuum state initially, and why that state had no (or at least few) correlations.

Page has emphasized the fact that de Sitter space is time-symmetric². As the inflationary phase is finite in duration, the patch of de Sitter space corresponding to this phase will have an 'in' and an 'out' region. According to the inflationary scenario, metric perturbations (irregularities) about the de Sitter background present in the in region will decay during the inflationary phase, and so will be infinitesimal in the out region. However, because of the time symmetry, for every solution of the gravitational field equations which corresponds to decaying perturbations, there will be a time-reversed solution which describes growing perturbations. (The proof of Boucher and Gibbons⁸ that the metric approaches de Sitter space exponentially fast assumes an infinite future with non-zero cosmological constant, thus breaking the time symmetry.) A sufficient, though not necessary, condition for decaying perturbations is the absence of correlations in the metric perturbations in the in region. (Roughly speaking, the perturbations will only grow if they conspire to organize themselves over a large spatial region in a cooperative fashion.) The situation is directly analogous to a Boltzmann-type model gas in a state of molecular chaos.

It is now necessary to explain why it is reasonable for the universe to have been in a state with no correlations initially. My argument is an adaptation of that frequently used³ to explain why randomly selected branch systems in low entropy states are exceedingly likely to be in a state close to molecular chaos.

To repeat the molecular argument first: imagine a gas enclosed for all time in an impenetrable box. The gas spends the overwhelming proportion of its time very close to thermodynamic equilibrium at maximum entropy $S_{\max}$, but occasionally makes small excursions from equilibrium on account of random fluctuations. The theory of fluctuations demands that small fluctuations, whilst rare, are nevertheless exceedingly more probable than large fluctuations.

Suppose now that the box of gas were to be equipped with a sensor that flashed a light whenever the gas achieve a state having a particular pre-selected value of the entropy, say S , where $S < S_{\max}$. It then follows that, on inspection, this state would almost certainly be very close to a local minimum in the entropy curve, close to the point of maximum excursion from $S_{\max}$. The reason for this is that there are vastly more fluctuations which just reach S from $S_{\max}$ than there are larger fluctuations that pass through S on their way to still lower values of the entropy. Hence a randomly selected low-entropy state is overwhelmingly likely to be at a local entropy minimum.

Now it may be shown that³ the condition of molecular chaos holds at the minimum point of an entropy fluctuation (which is, of course, a point of time symmetry with the entropy increasing in both forward and reverse time directions). It follows that a randomly selected low-entropy state will almost certainly be one essentially devoid of molecular correlations. This argument explains why it is that in a realistic physical situation, where a box of gas does not remain undisturbed for all time but instead is formed as a quasi-isolated branch system⁹ separated off from the wider environment, the entropy of the gas is always observed to rise. It is because, if the branch system is formed at random (and not at one of those rare instances where the molecules are conspiring to produce a large fluctuation to still lower entropy), it will almost certainly be in a state of no correlations (molecular chaos), for which the conditions of Boltzmann's H-theorem apply.

The case of de Sitter space is very closely analogous to the box of gas. de Sitter space may be considered to be a state of gravitational thermodynamic equilibrium, and indeed there is even an associated temperature¹⁰. (It may seem puzzling that the highly-symmetric de Sitter space is described as a maximum entropy state, whereas earlier it was stated that self-gravitating

systems tend to grow irregularities as they evolve towards thermodynamic equilibrium. The difference arises from the crucial role played by the cosmological constant. When such a constant is present the tendency for a self-gravitating system to grow clumpy is reversed; the exact opposite situation obtains, as explained in ref. 1.) Quantum effects will cause fluctuations to occur about the strict de Sitter background. Again, large fluctuations are much rarer than small fluctuations. At the minimum point of such a fluctuation the perturbations will be uncorrelated, corresponding to 'cosmic molecular chaos'. By the same reasoning as above, a randomly chosen perturbed state will almost certainly be such a state of no correlations at the minimum of a fluctuation curve. This state is then one in which the perturbations will decay rather than grow whichever direction of time is chosen as forward. Of course, in the inflationary scenario, a past-forward time convention is imposed by the very different nature of the in and out regions, the out region corresponding to a hot Friedman universe and the in region of a form to be discussed below.

Although the state of molecular chaos in a gas is sufficient for it to be shown, via the *H*-theorem, that entropy subsequently increases, such a state is not necessary. Indeed, it is easily demonstrated³ that if a gas is in a state of molecular chaos at some instant, it will not be in that condition at subsequent instants. Thus the *H*-theorem cannot be used to prove the continuous approach of a molecular system to equilibrium. Nevertheless we all *know* that the gas *will* so equilibrate, in spite of the correlations introduced during the climb towards maximum entropy as a result of the very molecular collisions that drive it there. Similarly, although a zero-correlation state is sufficient for the decay of perturbations in de Sitter space it is not necessary. Many states with some correlations will also tend towards de Sitter space. Thus there is a very much larger class of possible initial states consistent with the standard inflationary scenario.

To complete the inflationary picture it is necessary to have some idea of how the universe achieved its initial state, prior to the inflationary phase. At this point one must distinguish between two versions of the inflationary theory. In the first version the universe cools into the false vacuum from a pre-existing hot Friedman-like phase. While, as has been argued above, it seems likely that if such universe embarks upon inflation then any initial irregularities will decay, inflation will only proceed in any case if there is already a reasonable degree of regularity; inhomogeneity and anisotropy tend to suppress the inflationary mechanism¹¹. In spite of this, there will exist regions of space where by chance fluctuations the conditions are right for inflation to proceed. Thus, amid a sea of chaos, here and there bubbles of space will inflate. It only then remains for us to explain why we happen to be living in such a region. But it is only in the post-inflationary bubbles that large-scale order appears, itself an essential prerequisite for life and observers. We could not possibly exist amid the chaos outside of our quiescent 'oasis'. This is, of course, essentially a resurrection of Boltzmann's explanation of time asymmetry, but in a modern guise which circumvents the original objections for the following reasons.

First, the effect of inflation is to enormously amplify the initial chance fluctuation to moderate smoothness. The inflationary mechanism permits a fairly modest fluctuation to trigger the spontaneous growth of a huge ordered region containing a vast quantity of negative entropy relative to the locally maximum attainable entropy. Thus, inflation acts as a negative entropy amplifier. (The precise way in which this 'entropy gap' is widened by inflation has to do with the non-conservation of useful energy, and was explained in ref. 1.) This is in contrast to Boltzmann's original concept, in which the very large degree of negative entropy observed in our region of the universe today had to be generated *in toto* by a far less probable conjunction of fluctuations.

Second, in the context of the inflationary model, it appears that we do live in a minimum fluctuation region. It is a feature

of the inflationary theory that you either get a whole bubble universe or nothing. That is to say, once a modest fluctuation has triggered off inflation in a region, the dynamics of the inflationary mechanism will inevitably produce a bubble universe of extra-Hubble proportions. (The size of the fluctuation region is determined by the parameters of the theory, which fix such things as the 'roll-over' time¹².) Thus, a universe of the scale we observe is either produced in one blow, or not at all. So there is no mystery as to why a Hubble-scale fluctuation region represents a vast over-provision of negative entropy as far as life on Earth is concerned.

Finally, I turn to the other version of the inflationary scenario wherein the universe arrives at the threshold of inflation as the result of a quantum tunnelling process 'from nothing'. Vilenkin has proposed¹³ that the universe was 'initially' in a quantum state in which there is no classical spacetime, a state he refers to as 'nothing'. As a result of quantum fluctuations the universe then 'tunnels' into a de Sitter space from which inflation may proceed as usual. The tunnelling amplitude is discussed using a Euclideanized path integral for a quantum description of the gravitational field. Using semiclassical techniques borrowed from quantum mechanics and quantum field theory, Vilenkin argues that the most probable spacetime to emerge 'from the tunnel' is homogeneous and isotropic. Furthermore, the associated scalar field, which subsequently drives the inflationary mechanism, is found to be in the required false vacuum state. Although Vilenkin's original discussion was speculative and largely heuristic, his results have received support from the more detailed work of Moss and Wright¹⁴ on the wave function of the universe.

Similar ideas to those of Vilenkin have been proposed independently by Hawking¹⁵. The key element here is the suggestion that the quantum state of the universe might be one that is uniquely defined by a 'natural' mathematical prescription. This prescription has been discussed by Hartle and Hawking¹⁶, and in essence selects something akin to a minimum energy state of the universe. Hawking and Luttrell¹⁷ argue that the Hartle-Hawking ansatz requires an isotropic universe. Similar arguments can be used to select a homogeneous initial state (I. G. Moss, personal communication).

Thus, in this version, the universe is automatically driven towards something like de Sitter space by quantum processes, from which inflation proceeds in the usual way. Here it is not necessary to invoke an anthropic argument to select an ordered 'bubble' from the rest of the universe.

To what extent can these (necessarily imprecise) remarks provide a basis for an explanation of time asymmetry in the universe? The word 'explanation' can have two meanings. First it can mean 'tracing the origin'. Certainly the inflationary scenario provides a very plausible description of how the universe became 'wound up' as a result of quantum and gravitational processes. Once thus wound, the time asymmetry of the universe subsequently is assured, as explained in ref. 1.

But we can still ask why the universe started out in the required pre-inflationary state in the first place. This is a deep philosophical issue, for we are obviously in danger of falling into an infinite regress. Conceivably one could show quantum mechanically that the probability of producing a universe already in its maximum entropy state is exceedingly small. Indeed, it seems very plausible that a universe which originates from some sort of quantum gravitational fluctuation would be of Planck dimensions and Planck temperature, rather than Hubble dimensions and full of black holes. In the case of the version of inflation that starts from a Friedmann-like phase, this pre-inflationary state is usually taken to originate in a 'little bang' singularity, and the question of 'why that Friedmann-like state' reduces to the daunting (and perhaps unphysical) problem of what comes out of a spacetime singularity¹⁸.

In both versions, then, we have the difficulty that the chain of explanation is pushed back so far that it disappears off the edge of spacetime, either into a quantum tunnel or a singularity. Perhaps one has to stop there; after all, we can hardly make

nuch sense of the question 'why that state in the first place?' when there is no longer any classical spacetime for there to be a 'first place'. In this respect, the Hartle-Hawking approach to defining a unique state for the universe is attractive because it makes no use of the concepts of initial or final. Thus, we are forced to change the nature of the questions from the traditional 'why that spatial state in the first place?' to 'what is the most natural state of spacetime?'

My explanation for time asymmetry from inflation thus depends on which of the two alternative scenarios one considers. In the tunnelling version one adopts some criterion of 'naturalness' to select a very special initial state for the whole universe, in which case inflation merely acts to drastically reduce the improbability of such a state by permitting a wide class of initial states to develop into something like the universe we now see. In the other version, one can accept an arbitrary initial state but appeal to anthropic selection of an atypical region. Here, inflation is a crucial element in the argument because it explains why the fluctuation region necessary for observers to evolve is so large. In this sense, then, I maintain that inflation does explain time asymmetry.

I thank Don Page and Ian Moss for useful discussions.

Received 30 April; accepted 8 October 1984.

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Two new young galactic supernova remnants

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Substantial reductions¹⁻³ in the distances inferred for the remnants of several 'historical' supernovae, coupled with a galactic supernova rate of 1 every 25 yrs or so⁴, have raised the possibility⁵ that many young but distant galactic supernova remnants may be missing from present catalogues. We have now used the Very Large Array (VLA) and the Cambridge 5-km telescope to observe 32 small-diameter objects selected from galactic plane radio surveys. These observations reveal both a new young shell remnant (which, at only ~1 arc min in extent, is the smallest angular size galactic remnant yet discovered), and a very peculiar radio source, probably a supernova remnant only a few hundred years old, which is still in the free expansion phase.

Supernova remnants mark the violent endpoints of stellar evolution, and studies of them are relevant not only to our understanding of supernovae and pulsars, but also to that of the interstellar medium and the generation in fast shocks of relativistic particles and magnetic fields. These problems can be tackled either statistically or by detailed investigations of



Fig. 1 CLEANed map (500 components) of G1.9+0.3 at 4.9 GHz, restored with a 4.4×2 arc s² beam. The plotted range, black to white, is -1 to 9 mJy per beam.

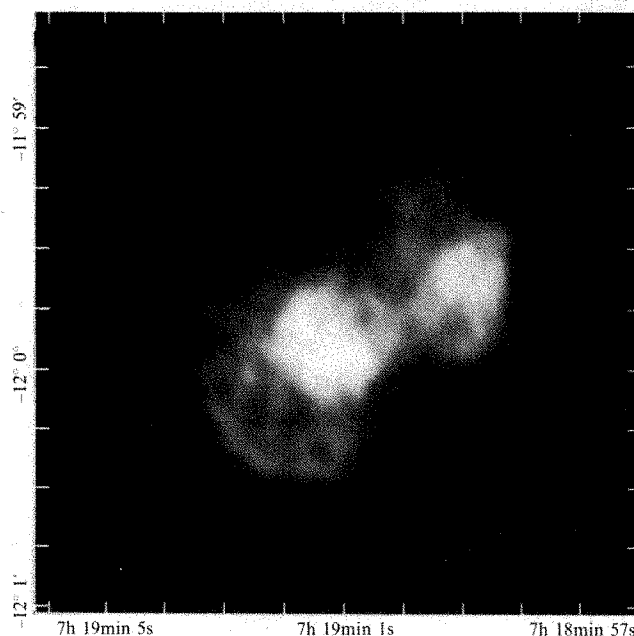


Fig. 2 CLEANed map (2000 components) of G227.1+1.0 at 4.9 GHz, restored with a 6×4 arc s² beam. The plotted range, black to white, is -1 to 9 mJy per beam.

individual remnants, among which the relatively bright remnants of historical supernovae (see ref. 6) are particularly important. Some of these, such as Cassiopeia A and the Crab Nebula, are amongst the brightest radio sources in the sky, so it is unlikely that any similar remnants in the Galaxy could have been missed. Others, such as 3C58 and the remnants of Tycho's and Kepler's supernovae, would have flux densities (at 1 GHz) of only ~1 Jy, and be <1 arc min in extent if they were removed to the other side of the Galaxy (~20 kpc). Present catalogues contain no such remnants, but existing galactic radio surveys, with only moderate resolution (a few arc minutes), do contain many unresolved or barely resolved sources with flux densities and spectral indices appropriate for young but distant supernova remnants. Few of these sources have been observed with higher resolutions.

Clark and Crawford⁷ list 513 small-diameter (<1.5 arc min) sources in the southern galactic plane ($|b| < 3^\circ$, declination below $+17^\circ$) from the Molonglo survey⁸, with flux densities >0.6 Jy at 408 MHz. Sources on this list which are brighter than 1 Jy (and

Table 1 Observed sources

Galactic coordinates		
<i>l</i>	<i>b</i>	Comments
Observed with the 5-km telescope at 2.7 GHz		
49.66	+2.91	Several sources*
51.22	-1.42	Single compact source
53.43	-1.28	Single compact source
193.63	-1.52	Close double
Observed with the VLA, 'C' array, at 5 GHz		
1.87	+0.33	Shell remnant, ~1 arc min in diameter
6.76	+0.92	Close double
9.82	-0.99	Single compact source
11.97	+0.19	Double, ~1 arc min, with bright core
20.71	+2.29	Single compact source
21.05	+1.96	Single compact source
25.26	-0.14	Three sources
24.88	-1.71	Close double
32.26	+1.17	Single compact source
32.95	-1.79	Close double
39.56	-0.04	Single compact source
39.42	-0.59	Close double
197.14	-0.85	Close double
201.07	-1.02	Single compact source
203.52	-1.40	Single compact source
201.53	+0.51	Single compact source
214.70	-1.75	Close double
217.78	-2.95	Single compact source
213.13	+0.57	Double, ~1 arc min
223.54	-2.82	Close double
226.52	-2.92	Single compact source
224.66	-1.72	Single compact source
229.87	-0.64	Single compact source
230.08	-0.62	Single compact source
227.09	+1.02	Strange source, ~1 arc min in extent
238.40	-2.46	Single compact source
237.32	-1.77	Single compact source
245.16	+2.77	Single compact source

* Also observed by Helfand *et al.*¹⁰.

some of the fainter ones) have been observed further⁹ with the Parkes 64-m radio telescope at 5 GHz, so that spectral indices are available (with errors <0.1) for 325 of these small-diameter sources. From these lists we selected candidate young supernova remnants on the criteria that the declination should be above -30° (so that reasonable maps could be made with the VLA), and the spectral index, α (where $S(\nu) \propto \nu^{-\alpha}$), should be between 0.0 and 0.8, which covers the expected range for both filled-centre ($\sim 0.2 \pm 0.2$) and shell-type ($\sim 0.6 \pm 0.2$) remnants. (Three other sources, each with flat spectra ($\alpha < 0.4$), satisfy these criteria, but these have already been observed by Helfand *et al.*¹⁰ in their unsuccessful search for new young 'filled-centre' remnants.)

Table 1 lists the galactic coordinates (from ref. 9) of the 32 sources we have observed. The four sources above declination +15° were observed in April 1984 with the Cambridge 5-km telescope at 2.7 GHz, with a resolution of $\sim 4 \times 14$ arc s (ref. 2), and the other 28 were observed in May 1984 with the VLA in C configuration at 4.9 GHz, with a resolution of ~ 4 arc s. Most of these objects are compact single or close double sources, and are presumably extragalactic. Four of them are, however, extended on a scale of ~ 1 arc min: two are extended double sources and are probably also extragalactic. The other two extended sources are discussed below.

G1.9+0.3 (Fig. 1) is a shell source with a radio surface brightness of $\sim 7 \times 10^{-20} \text{ W m}^{-2} \text{ Hz}^{-1} \text{ sr}^{-1}$ at 1 GHz, which is slightly less than those of Tycho's and Kepler's supernova remnants, but appreciably larger (by a factor of 25) than that of the remnant of SN1006. Its radio spectral index is ~ 0.7 , comparable with that of known young shell remnants. It is thus

almost certainly a supernova remnant. Although this source is nearly circular, the intensity of radio emission varies considerably around the shell and is strikingly similar to Kepler's supernova remnant¹¹ in this respect. A maximum galactocentric radius of ~ 20 kpc gives an upper distance limit of ~ 30 kpc for this object, which then implies a diameter of < 10 pc. (Compare this with Tycho, Kepler and SN1006 supernova remnants, with diameters of 5, 4 and 9 pc, respectively.) We conclude that the age of this remnant is between 400 and 1,000 yr.

G227.1+1.0 (Fig. 2) is a very peculiar source, unlike known galactic supernova remnants or extragalactic sources. Its surface brightness is $\sim 2 \times 10^{-19} \text{ W m}^{-2} \text{ Hz}^{-1} \text{ sr}^{-1}$ at 1 GHz, which is similar to that of known young remnants such as those of Tycho and Kepler and 3C58. Its radio spectral index is ~ 0.6 , and the non-thermal nature of the radio emission is confirmed by its high degree of polarization (Fig. 3).

If this is a galactic object, it is likely to be closer than ~ 10 kpc, because its galactic longitude is 227° , towards the galactic anti-centre. Its linear size would then be < 4 pc, consistent with a supernova remnant only a few hundred years old.

Our interpretation of this source is that it is the result of a very recent supernova, at most a few hundred years old. The slightly limb-brightened lobes of fainter emission to the east and west would be a ring of ejecta from the supernova explosion which is beginning to be decelerated by its interaction with the surrounding interstellar medium. Furthermore, if the Faraday rotation to the source is small at 5 GHz, then the magnetic field alignment is tangential to the edge of the emission in the eastern lobe, as might be expected. The brighter central plateau of emission is, in this interpretation, a young 'filled-centre' remnant, a plasma bubble powered by a pulsar, the compact remnant of the supernova explosion (which may be related to the one of the compact features visible in the brighter region in Fig. 2).

If both objects are young but distant galactic supernova remnants, their parent supernova may have been observed historically, and they should also be observable as X-ray sources. However, there are no historical records of 'guest stars' (see ref. 6) in the direction of either object, and neither is there a catalogued X-ray source¹².

The search for and detection of new young galactic supernova remnants described above confirms that present catalogues are incomplete with respect to young but distant remnants. Until this is rectified, statistical studies of galactic supernova remnants will be severely limited. On the other hand, the detection of further young supernova remnants will provide more objects that are relatively bright and easily studied.

We thank the staff of both the 5-km telescope and the VLA for their help with the observations. The VLA is operated by Associated Universities Inc., under contract with the NSF.

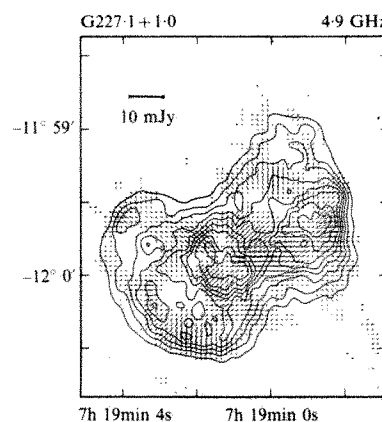


Fig. 3 CLEANed map of G227.1+1.0 restored with a 6×4 arc s² beam. Contours of Stokes parameter *I*, at $0.84 \times (1, 2, \dots, 10)$ mJy per beam with polarization *E*-vectors superposed where the polarized intensity exceeds 0.1 mJy per beam.

Received 10 September; accepted 8 October 1984.

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Hyperbolic umbilic diffraction catastrophe and rainbow scattering from spheroidal drops

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The characterization of short-wavelength scattering phenomena has been advanced by the study of diffraction catastrophes^{1,2}. Examples include optical phenomena¹⁻⁵ and molecular collisions⁶. The most familiar example of a diffraction catastrophe is the ordinary rainbow⁷⁻⁹. The angular variation of the scattering from a spherical drop locally corresponds to that of a fold-diffraction catastrophe^{1,2}, a result most clearly seen with monochromatic illumination. We have studied the scattering from drops whose shape closely approximated that of an oblate spheroid with the short axis—the symmetry axis—vertical. The drops were illuminated by a horizontally propagating gaussian beam with a wavelength $\lambda = 633$ nm. These drops were observed to scatter in the horizontal rainbow region with patterns like those of hyperbolic-umbilic (classification D_4^+) diffraction catastrophes^{1,5,6}. Visible D_4^+ diffraction patterns observed previously include light transmitted by frosted glass surfaces¹⁰ and by liquid lenses clinging to tilted glass plates¹⁴.

Figure 1 shows the ray diagram of the primary rainbow of a spherical drop. For the once-reflected (twice-refracted) rays there is a minimum scattering angle of $\theta_R \approx 138^\circ$ and the associated ray is known as the Descartes ray. When the scattering angle θ is between θ_R and 166° , there are two rays of the class shown. The rays labelled 1 and 2 are for $\theta = 152^\circ$. In the two-ray region, intensity oscillations produce the supernumerary arcs⁷⁻⁹. Figure 1 is also applicable to rays which lie in the horizontal equatorial plane P of the spheroidal drops; P cuts the drop's surface in a circle and both the incident ray and the local surface normals lie in P.

In our apparatus (see Fig. 2), a horizontal metal plate was coupled to a piezoelectric vibrator which was driven at a frequency of 27 kHz. A curved reflector (not shown in Fig. 2) was placed above the plate's centre so as to establish an acoustic standing wave in the air below the reflector. Drops of distilled water could be levitated in this wave by an upward-directed acoustic radiation pressure. (The levitator is similar, in principle, to one described in ref. 11.) Figure 3 shows a levitated drop. The oblate shape is due to the spatial distribution of the radiation pressure¹². In addition to the deformation evident in Fig. 3, the drop vibrates at 27 kHz with surface displacements which are too small to affect the light scattering. The drop's diameter will be denoted by D in plane P, and by H along the

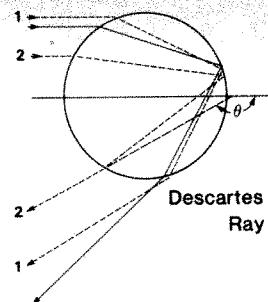


Fig. 1 Rays through either a spherical drop or through a spheroidal drop in the equatorial plane.

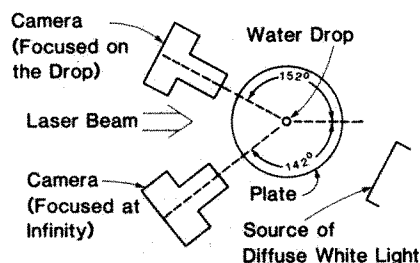


Fig. 2 Schematic view of the apparatus from above. The drop is held 11 mm above the plate and the optical axes of the cameras lie near a horizontal plane containing the drop. The diffuse light is usually turned off.

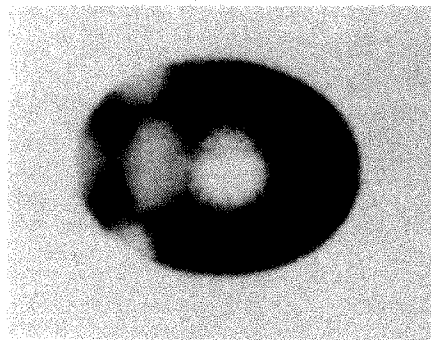


Fig. 3 Photographs of an acoustically levitated drop having a diameter $D = 2.3$ mm. The drop was illuminated both with diffuse light and the laser beam. The four patches on the left are due to refracted laser light.

vertical symmetry axis. Raising the ultrasonic amplitude increases D/H .

The scattering was photographed for randomly polarized illumination from a He-Ne laser. The camera was focused on infinity so that the pattern recorded was equivalent to that in the far field (which is at distances $r \gg D^2/\lambda$ from the drop). Horizontal and vertical coordinates in each photograph were linear in the horizontal and vertical scattering angles θ and β , where β is relative to plane P. The camera viewed the region: $136^\circ < \theta < 148^\circ$, $-6^\circ < \beta < 6^\circ$.

Figure 4a shows the scattering from a slightly nonspherical drop having $D = 1.9$ mm. The intensity modulations are similar to other photographs of supernumerary arcs with laser illumination¹³. Within experimental uncertainties, their spacing is as predicted by Airy theory^{7,9}. Figure 4b-f are the patterns for another less nearly spherical drop. For Fig. 4b the drop had $D = 1.39$ mm and $D/H \approx 1.23$; smaller values of D/H gave patterns similar to Fig. 4a. Changes in the pattern with changes in D/H were also viewed through the camera's lens by eye.

Figure 4d manifests the parallelogram and caustic structures characteristic of focal section patterns of D_4^+ diffraction

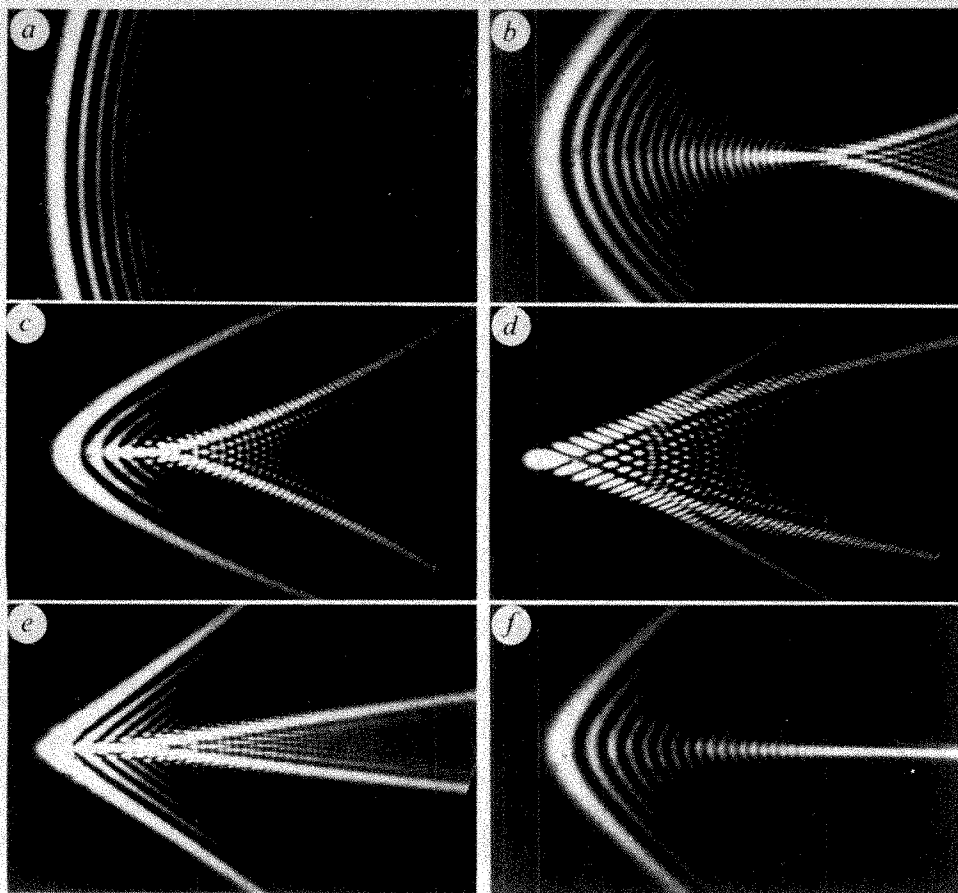


Fig. 4 Photographs of rainbow region scattering patterns arranged in order of increasing drop axis ratio D/H . The horizontal scattering angle θ increases from left to right. The cusp in *b* moved into view from the right as D/H was increased. *d* is the focal section of a hyperbolic-umbilic diffraction catastrophe while *c* and *e* manifest the unfolding of the catastrophe in response to small changes in D/H . Scale bar, 5° .

catastrophes. Consequently, the normal form^{1,2} of the associated diffraction integral has the control parameter $C_3 = 0$. Evidently the transverse control parameters C_1 and C_2 are locally related to β and $\theta - \theta_R$ by a linear transformation. The parallelogram structure is sheared with an apex angle $\approx 43.5 \pm 1^\circ$. Figure 4*b*, *c*, *e* and *f* correspond to sheared and scaled sections of D_4^+ patterns having $C_3 \neq 0$. Inspection of the patterns of the elementary catastrophes^{1,4} confirms that only the D_4^+ describes our observations.

When $C_3 \neq 0$, the D_4^+ pattern may be partitioned into three angular regions according to the number of stationary points of the phase function within the diffraction integral^{1,5,6}. The number of points corresponds to the number of once-reflected (twice refracted) rays which contribute to the scattering. This partitioning is shown in Fig. 5*a* for the pattern in Fig. 4*c*. That the right-most region has four rays was confirmed by direct observation. A camera having a small aperture, positioned as shown in Fig. 2, was focused on the drop. Figure 5*b* is drawn from a representative image so as to show the locations of the four observed rays. Rays 1 and 2 lie in plane *P* and correspond to those in Fig. 1. Rays 3 and 4 are horizontally directed, having $\beta = 0^\circ$ when outside the drop. They are skew rays because inside the drop they do not lie in a horizontal plane. These rays were not anticipated from previous studies^{13,14} of rainbows of spheroidal drops, which considered only rays confined to a plane.

Other features observed were consistent with those expected of a D_4^+ diffraction catastrophe at short wavelengths $\lambda \ll D$. The axis ratio D/H which gives $C_3 = 0$ should be independent of D for spheroidal drops having the same refractive index. To check this prediction, drops were illuminated with diffuse white light (see Fig. 2) and their profiles were photographed. Before each photograph, D/H was adjusted so that the laser scattering pattern was a focal section as in Fig. 4*d*. Photographs of six drops having D of 1.5–2.4 mm gave axis ratios not correlated with D . The average measured D/H of 1.305 ± 0.016 is a lower

limit of the true axis ratio because any misalignment of the camera's optical axis from the equatorial plane would give a systematic reduction in the measured D/H .

For scattering from large spherical drops, Airy's theory^{1,7} predicts that when $\theta = \theta_R$, the intensity I at a distance r has $I \propto (D/\lambda)^{1/3} (D/r)^2$. Examination of the D_4^+ diffraction integral^{1,5,6} gives $I \propto (D/\lambda)^{2/3} (D/r)^2$ when C_3 , β and $\theta - \theta_R$ all vanish. Visual inspections and photographs confirm that the scattering near the apex of the focal section appears brighter than that near θ_R of the other sections such as Fig. 4*b* and *c*. This focusing for $C_3 = 0$ may be observable in nature when the Sun is close to the horizon. Raindrops having $D \approx 4$ mm have an axis ratio ≈ 1.3 . However, such large drops are flattened below the centre¹⁵ and the effect on the focusing is not known.

These experiments were carried out while P.M. was at the Jet Propulsion Laboratory on a sabbatical leave. The research was partially supported by NASA and by the Office of Naval Research.

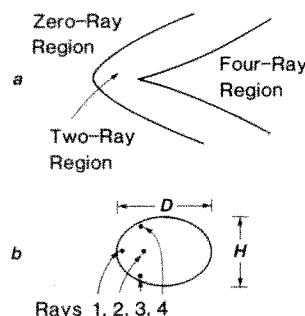


Fig. 5 *a*, Partitioning of the angular regions implied by the theory of hyperbolic-umbilic diffraction catastrophes for sections having $C_3 \neq 0$. *b*, Ray locations evident from the patches in Fig. 3. The camera's aperture was in the four-ray region.

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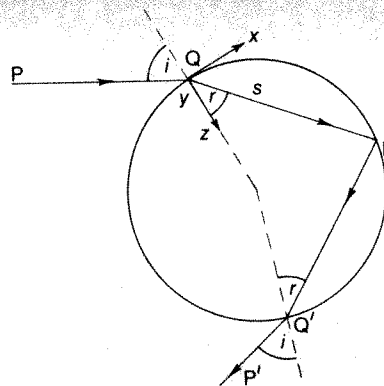


Fig. 1 The equatorial (horizontal) section of a spheroidal water drop showing the critical ray of minimum deviation.

Rainbow scattering from spheroidal drops—an explanation of the hyperbolic umbilic foci

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An experiment by Marston and Trinh¹, showed how an oblate spheroidal water drop illuminated by a parallel beam of light forms a caustic in the far field characterized by the hyperbolic umbilic catastrophe—a generalization of the primary rainbow formed by a spherical drop. As the axis-ratio of the drop was changed, they observed a singular section of the catastrophe appearing for $D/H = 1.305 \pm 0.016$ — D being the diameter in the horizontal equatorial plane and H the diameter along the vertical axis of rotational symmetry. I show here how this ratio may be calculated to confirm quantitatively the arguments¹ that the hyperbolic-umbilic catastrophe is associated with skew rays, and predict the occurrence of novel 'lips' phenomena, yet to be observed.

PQR'P' is the critical (Descartes) ray in the equatorial plane (Fig. 1). Keeping the incident direction fixed, if we were to move the entry point away from Q, but still in the equatorial plane, standard rainbow theory would then tell us that the resulting angle of deviation is a minimum for entry at Q—the angles of incidence, i , and refraction, r , are given by $\sin i = \sqrt{\{(4 - \mu^2)/3\}}$, $\sin r = \sqrt{\{(4 - \mu^2)/3\}\mu^2}$ and are connected by Snell's law $\sin i = \mu \sin r$, where μ is the refractive index.

If we now vary the entry point in the perpendicular (vertical) plane, still keeping the incident direction fixed, the once-refracted ray will, in general, pass above or below R by a first-order amount. However, we can show that, for a critical value of D/H , it continues to pass through R, to first order, and therefore emerges (by symmetry) displaced from the equatorial plane but without change of deviation, to first order. That is, we have directional focusing in the vertical as well as in the horizontal plane. Such simultaneous focusing in two directions gives rise to what is called, in catastrophe optics, an umbilic caustic. At R there is a horizontal focal line.

To find the critical D/H take the axes as shown, let the point of entry move vertically by ϵ , and assume that the refracted ray passes through R. To first order the direction cosines of the incident ray, the normal to the surface and the refracted ray are the rows of the determinant

$$\begin{vmatrix} \sin i & 0 & \cos i \\ 0 & \varepsilon/\rho & 1 \\ \sin r & \varepsilon/s & \cos r \end{vmatrix}$$

where $s = QR = D \cos r$ and ρ is the radius of curvature of the surface in the vertical plane, $\frac{1}{2}H^2/D$.

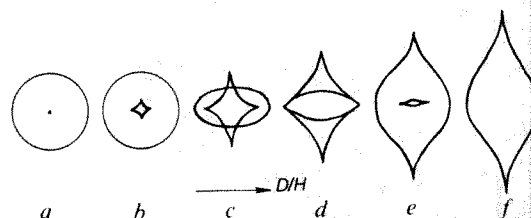


Fig. 2 Sequence of caustics in the far field as D/H increases from 1. *a*, The circular rainbow with a singular point at its centre. On perturbation the point breaks up (*b, c*) into an expanding four-cusped figure. At *d*, two hyperbolic umbilic foci occur. On further increase of D/H , the inner figure contracts (*e*), and then disappears (*f*) in a lips event. The angular width of the complete figure is the same throughout.

Snell's law is satisfied to first order without further conditions, but for the three directions to be coplanar the above determinant must be zero. A short calculation then yields the surprisingly simple result

$$\frac{D}{H} = \sec r = \sqrt{\frac{3\mu^2}{4(\mu^2 - 1)}}.$$

Putting $\mu = 1.3317$, the refractive index of water at the wavelength used in the experiments ($\lambda = 633 \text{ nm}$), gives $D/H = 1.3114$, which agrees with the observed value of 1.305 ± 0.016 . This is a stringent test that the shape of the drop is spheroidal.

I now show, using the principles of catastrophe optics, that the global topology of the caustic is that sketched in Fig. 2, taken from Hannay's work in appendix 2 of ref. 2. First notice that as $\mu \rightarrow 2$, the above critical ratio $D/H \rightarrow 1$ and that both i and r approach zero. That is, the caustic structure collapses into a single point in direction space (backwards)—the perfect 'catseye' reflector. Now unfold (that is, perturb) this degenerate focus by treating $(2 - \mu)$ and D/H as control variables; keeping $D/H = 1$ and varying μ from 2 causes the familiar rainbow circle to grow from the central point (Fig 2a). Now keep μ fixed and increase D/H : the sequence is then Fig 2a to f with two hyperbolic umbilics appearing symmetrically in the equatorial plane in Fig 2d. These are the ones calculated above.

The lips event occurring between Fig. 2e and f is at $D/H = \sqrt{\{\mu/2(\mu-1)\}} = 1.4168$ for the above value of μ . (There appears to be a second horizontal lips event on the axis at $D/H = \sqrt{\{(2\mu-1)/2(\mu-1)\}} = 1.5835$, not part of this catastrophe and arising from rays that pass through the centre of vertical curvature of the far side of the drop.)

Equation (A2.2) in ref. 2 shows that the form of the wavefront that emerges from the drop in the most singular case (spherical drop with $\mu = 2$), specified by its deviation $f(x, y)$ from a plane, is, to lowest order, $f = A(x^4 + 2x^2y^2 + y^4)$, which is a singularity of infinite codimension; that is, there are an infinite number of unfolding terms. However, the symmetry-breaking perturbation

from sphere to spheroid, with $\mu \neq 2$, yields a structurally stable caustic.

To establish directly that the umbilics calculated above are indeed hyperbolic rather than elliptic would have required a complicated analysis of the cubic terms in the expression for the wavefront as it was refracted and reflected astigmatically through the spheroidal water drop. But recognizing that the umbilics are part of the unfolding of a higher catastrophe removes the need for this calculation, for there is no reason to suspect that they change their character when the unfolding is finite rather than infinitesimal, as assumed in the theory. Note, however, that I have calculated the critical D/H ratios exactly, rather than merely to lowest order in $2-\mu$ and $D/H-1$.

I thank Drs Marston and Trinh for showing me their letter before publication.

Received 14 September; accepted 6 November 1984.

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A very narrow Mössbauer resonance in ^{63}Ni

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Investigations of the Mössbauer effect in nickel have been less fruitful than those on iron, because until now there has been no convenient resonance akin to the 14-keV transition (half-life 98 ns) in ^{57}Fe . So far the only available Mössbauer resonance in nickel has been the 67-keV transition (half-life, 5 ns) in ^{61}Ni , whose natural resonance line is so broad that its width is comparable to the nuclear Zeeman splitting in ferromagnetic nickel metal. The result is a spectrum comprising a single broad line instead of a well-resolved splitting into discrete lines, as is the case for narrow resonances. We have recently succeeded in a scattering experiment in observing the Mössbauer effect in the 87-keV transition (half-life 1.7 μs) in radioactive ^{63}Ni ($t_{1/2} = 100$ yr). Because of the long lifetime of this state, its natural linewidth is smaller than that of ^{61}Ni ($\times 330$), and the commonly used ^{57}Fe ($\times 17$), but still larger than that of ^{67}Zn ($\times 0.2$), which has the highest relative energy resolution of all Mössbauer resonances in use. The use of ^{63}Ni should thus permit us in future to perform detailed Mössbauer studies in nickel.

The γ -source for observing the Mössbauer effect in ^{63}Ni is ^{63}Co (half-life 27.5-s), produced by the $^{64}\text{Ni}(t, \alpha)^{63}\text{Co}$ reaction with the triton beam of the Technical University of Munich Compact Cyclotron¹. With this reaction, ^{63}Co could be produced essentially free of unwanted radioactivity, which is not the case when using other projectiles. A production rate for ^{63}Co of ~ 1 mCi min^{-1} was possible during irradiation with a triton beam of ~ 2 μA .

A total of 15 targets were used to avoid accumulation of long-lived activity by repeated irradiation. The target material was metallic nickel foil of 27 mg cm^{-2} thickness and 10 mm diameter, made of enriched (97.7%) ^{64}Ni . These foils were soldered to coin-shaped target holders made of copper disks 6 mm thick and 25 mm in diameter. A hole of 10-mm diameter was bored along the symmetry axis of the discs, leaving a wall-thickness of 0.5 mm at the bottom. The ^{64}Ni foils were soldered using lead solder into this cylindrical cavity under vacuum conditions. Before irradiation the targets were cooled to liquid nitrogen temperature. Because of the energy loss of the tritons in the target foil, we are sure that the temperature of the ^{64}Ni is higher than 77 K during irradiation presumably allowing annealing of radiation damage in the nickel lattice to

occur. After irradiation, the heat dissipated in the nickel foil is absorbed owing to the high heat capacity of copper target holder, thus rapidly cooling the target foil again to the temperature of liquid nitrogen. The sources were then quickly transported, within 6-8 s, to a measuring position, well-shielded from the accelerator. This was accomplished by rolling the targets through inclined tubes of rectangular cross-section. The absorber and scatterer was a ^{63}Ni foil (12% ^{63}Ni , 15-mm diameter, 0.2-mm thickness), which was also cooled to liquid nitrogen temperature.

We have used 41 mg of ^{63}Ni corresponding to 2.3 Ci of the 100-yr half-life activity. Ferromagnetic nickel metal was chosen as the chemical form of source and absorber (scatterer), and both are thus subject to a magnetic nuclear Zeeman splitting. Only the unshifted central resonance line was covered by the velocity drive. The relative Doppler velocity between the source and the absorber (scatterer) was obtained by moving the absorber sinusoidally with a piezo-drive. The maximum velocity was 35 $\mu\text{m s}^{-1}$ and the velocity drive was calibrated by observing the well-known isomer shift between $^{67}\text{GaCu}$ and $^{67}\text{Zn-}\beta$ -brass. The scattered and transmitted radiation of the sources were measured simultaneously by two planar intrinsic germanium detectors and recorded by two multichannel-analysers synchronously with the velocity of the drive.

After more than 200 measuring cycles (altogether 10 h of measuring time) a resonance line was seen in the scattering spectrum with a linewidth of 5 times the minimum resonance width, having taken the scatterer thickness into account. This spectrum is shown in Fig. 1. Its intensity corresponded very well

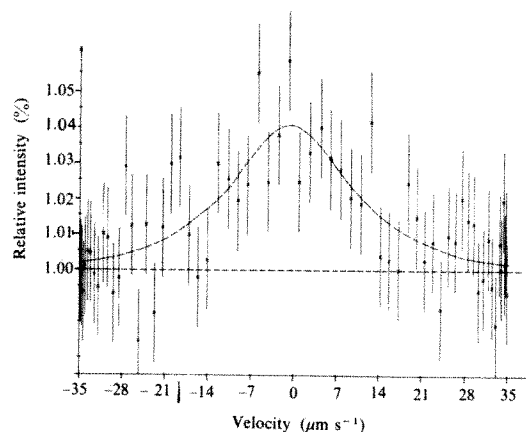


Fig. 1 Intensity of the recoil-less scattered radiation from a ^{63}Ni scatterer. (Only the central resonance line is observed.)

with the value calculated by applying to the data the procedure of Debrunner and Morrison². This indicates that the adopted Debye-Waller factor for liquid nitrogen temperature, derived from ^{61}Ni experiments, reproduces the actual intensity very well. The resonance line disappeared when the temperature of source and scatterer was raised to room temperature. However, the statistics were still not good enough to observe the resonance in transmission geometry. The broadening of the line is most probably due to radiation damage in the source material.

We shall now try to reduce the linewidth and find a compound that gives an unsplit single emission line without generating too much unwanted radioactivity. Also certain basic experiments, such as the determination of the ground state magnetic moment of ^{63}Ni , are being planned.

We thank the Deutsche Forschungsgemeinschaft for their support.

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Structure of Theta-1, the first unidimensional medium-pore high-silica zeolite

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Synthetic high-silica zeolites (typified by ZSM-5) are powerful catalysts used extensively in the petroleum industry. The channels and cavities of their aluminosilicate framework render their catalytic activity very shape-specific. The varying attributes of individual members of this family depend primarily on subtle changes in framework topology. Unfortunately, these high-silica zeolites are difficult to synthesize as large, that is $>100\text{ }\mu\text{m}$, single crystals, so conventional X-ray structural methods are not appropriate. We have used a combination of techniques to elucidate the framework topology of a new, synthetic high-silica zeolite, Theta-1, which was first prepared as small $<2\text{ }\mu\text{m}$ crystals in our laboratories. It exhibits a novel unidimensional 10-T-ring channel system with a medium pore structure, thereby differing from other known structure-types that classify zeolites and suggesting unique shape-selective properties.

Crystals of Theta-1 were synthesized as described previously^{1,2} and exhibited rod-like morphology, being $\sim 0.1\text{ }\mu\text{m}$ in diameter and $\sim 2\text{ }\mu\text{m}$ in length. For characterization, samples were converted to the hydrogen form using standard methods^{1,2}. The bulk $\text{SiO}_2:\text{Al}_2\text{O}_3$ molar ratio was determined by X-ray fluorescence

indexed on the basis of a C-centred orthorhombic unit cell (uc) giving the dimensions $a = 13.836 \pm 0.003$, $b = 17.415(4)$ and $c = 5.042(1)\text{ }\text{\AA}$ by refining 28 calibrated and measured lines.

Structural analysis followed these multi-technique data. A framework density of 20.5 tetrahedral-atoms (T-atoms) per $1,000\text{ }\text{\AA}^3$ was calculated⁶ from the crystal density of 2.05 g cm^{-3} measured by mesitylene displacement. Adsorption (Table 1) showed a maximum void fraction (V_f) 0.21 which also gave⁶ 20.5 T-atoms per $1,000\text{ }\text{\AA}^3$. These translated to 24.9 T-atoms per uc in good agreement with a minimum site multiplicity of 4 (that is 24 T-atoms per uc) for each of the three space groups⁷. Trial structures for Theta-1 were evaluated by generating sufficient possible Fourier projections in the a - b plane from experimentally-measured integrated intensities. (Such a method is only possible for centrosymmetric projections down short axes, such as the $5.04\text{-}\text{\AA}$ axis of Theta-1, where atomic overlap is minimized.) Full-matrix least-squares refinement of one trial structure having the $\text{Cmc}2_1$ space group successfully converged to $R = 0.13$ for 302 reflections ($2\theta \leq 90^\circ$). The framework topology of this structure is shown in Fig. 1A, and the refined atomic coordinates are listed in Table 2. The mean of the seven independent Si-Si distances was $3.10(7)\text{ }\text{\AA}$ in a range $3.01\text{--}3.24\text{ }\text{\AA}$ with all distances lying within the 2σ limit. The SiO_4 tetrahedral geometry was suitable. The range of 14 independent Si-O distances was between 1.50 and $1.65\text{ }\text{\AA}$ with a mean of $1.59(6)\text{ }\text{\AA}$. The mean O-Si-O tetrahedral angle was $109(4)^\circ$ in the range $102\text{--}118^\circ$.

The proposed structure is rich in 5-T rings, as is expected for high-silica tectosilicate structures⁵, and can be constructed from complex 5-1 secondary building units O^- (from ref. 8). This places the proposed structure in the Mordenite (Pentasil) group

Table 1 Adsorption properties of Theta-1

Adsorbate	Kinetic diameter ($\text{\AA}$)	V_p ($\text{cm}^3\text{ g}^{-1}$)	V_f
N_2	3.64	0.1	0.21
H_2O	2.65	0.058	0.12
<i>n</i> -hexane	4.3	0.089	0.18
<i>p</i> -xylene	5.85	0.062	0.13
Cyclohexane	6.0	0.021	0.04
<i>m</i> -xylene	6.0	0.031	0.06

Void volume V_p ($\text{cm}^3\text{ g}^{-1}$) is the total micropore volume in hydrogen form of Theta-1, calculated using normal liquid density at adsorption temperature. The void fraction, $V_f = V_p$ ($\text{cm}^3\text{ g}^{-1}$) $\times d_c$ (g cm^{-3}), where d_c ($=2.05\text{ g cm}^{-3}$) is the measured crystal density. All samples activated by calcination at 550°C for 1 h. Adsorption measurements by microbalance techniques at room temperature with relative pressure $P/P_0 = 0.5$. N_2 adsorption by single point Brunauer-Emmett-Teller test, -183°C $P/P_0 = 0.9$.

(XRF) and confirmed by magic angle spinning NMR (MASNMR)³. Typical XRF values were 60:1. ^{29}Si MASNMR spectra showed peaks of relative area 13.9:1 at -113 and -105 p.p.m. relative to tetramethylsilane, confirming^{4,5} a tectoaluminosilicate with a 60:1 $\text{SiO}_2:\text{Al}_2\text{O}_3$ ratio. This was supported by a single ^{27}Al peak at ~ 57 p.p.m. relative to $\text{Al}(\text{H}_2\text{O})_6^{3+}$, indicative of tetrahedral Al. The adsorption properties of Theta-1 were measured with a range of adsorbates to probe shape-selectivity (Table 1). The results show that the zeolite is microporous and that access to the pore structure becomes restricted at $6\text{ }\text{\AA}$.

The structure of Theta-1 was studied by the diffraction of both electrons and X rays. Selected-area electron diffraction patterns show orthorhombic symmetry and reveal unit cell parameters with a short c -axis repeat value of $5\text{ }\text{\AA}$ which is particularly significant in zeolites. In addition, systematic absences are consistent with three possible space groups, namely Cmcm , $\text{C}2\text{cm}$ and $\text{Cmc}2_1$. The X-ray powder diffraction pattern was

Table 2 Fractional atomic coordinates ($\times 10^3$)

Atom	x	y	z
Si(1)	283.8 (9)	50.3 (7)	250*
Si(2)	201 (1)	215.7 (8)	312 (4)
Si(3)	500†	218 (1)	824 (6)
Si(4)	500†	118 (1)	320 (7)
O(11)	264 (2)	-10 (2)	493 (8)
O(12)	214 (2)	125 (2)	309 (8)
O(14)	404 (2)	65 (1)	280 (9)
O(22)	257 (3)	257 (2)	534 (7)
O(23)	407 (2)	269 (2)	795 (10)
O(34)	500*	152 (3)	628 (10)
O(43)	500*	187 (2)	1,132 (9)

* Figures in parentheses give $\pm$ s.d.

† Fixed parameter.

of zeolite structures⁵. These include mordenite, ferrierite, dachiardite, epistilbite and bikitaite (ZSM-5 and ZSM-11). A projection in the a - b plane shows zig-zag chains of edge-shared 5-5-T-rings (Fig. 1B) in the a direction linked by 6-T-rings in the b direction to form elliptical 10-T-ring channels running along the c direction. The same structural feature is found in ferrierite, ZSM-5 and ZSM-11 (ref. 9), although with different specific topology. In contrast, in the c direction, projections strongly resemble comparable projections in bikitaite^{5,10} and consist of interlinked 6-T-ring sheets. Thus, like bikitaite, the proposed topology has a unidimensional channel system. The presence of 5-T-rings in Theta-1 is confirmed by IR spectroscopy. A band at $1,230\text{--}1,210\text{ cm}^{-1}$ has previously¹¹ been assigned to this structural arrangement.

V_f values for adsorbate molecules of different size and shape (Table 1) indicate a pore diameter of $\sim 6\text{ }\text{\AA}$ similar to that found for ZSM-5. However, ZSM-5 has two intersecting 10-T-ring structural channels and a maximum V_f of 0.32. The much lower value of 0.22 for Theta-1 argues for a unidimensional 10-T-ring channel system, also made necessary by the very short c -axis

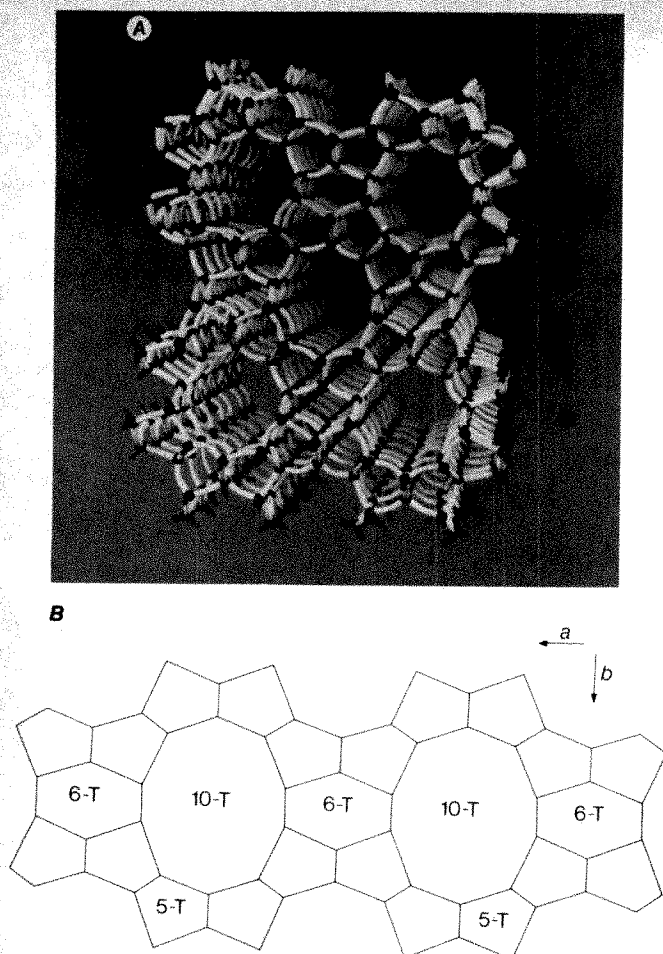


Fig. 1 A, Theta-1 framework viewed down axis *c*. This projection is similar to the *a-c* projection in the zeolite Bikitaite. B, Projection in the *a-b* plane of 5-5-T and 6-T rings to show the elliptical 10-T ring channels in the *c* direction.

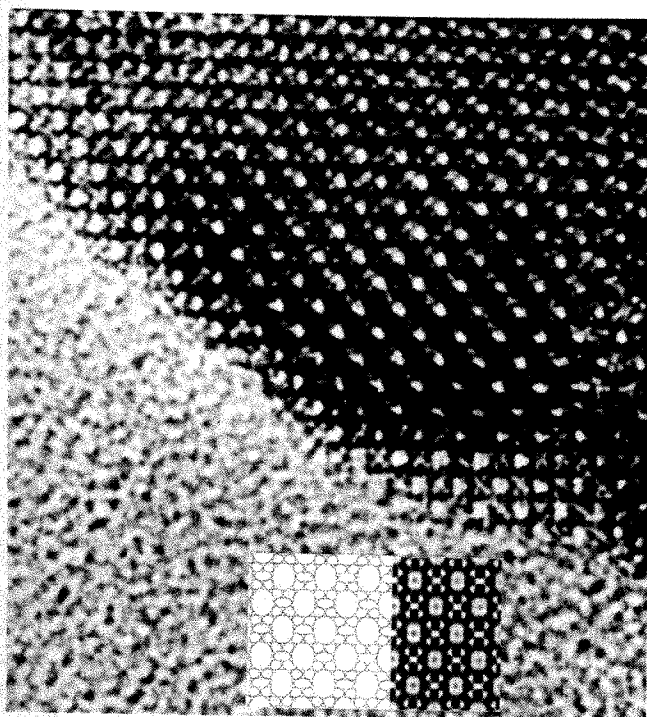


Fig. 2 High-resolution transmission electron micrograph in the *a-b* projection. Insets show schematic proposed structure (left) and calculated image (right).

(5.042 Å). The ^{29}Si MASNMR of a Theta-1 aluminosilicate framework, dealuminated by calcination at 800°C, showed closely positioned Si(OA1) peaks at -110.5, -112.4 and -113.9 p.p.m. The broad width of the peak at -110.5 p.p.m. (2.5 compared with 1.9 p.p.m.) strongly suggests a composite of two peaks at very similar chemical shifts, or a minimum of 3 or 4 crystallographically-distinct T-atoms. Using the relative peak areas, framework density, and space-group site multiplicity restrictions³, we obtained relative site occupancies of 8:8:8 or 4:4:8:8 per uc. The proposed structure has four crystallographically-distinct T-atoms of relative occupancy 4:4:8:8. The two four-occupied sites are topologically very similar and would be expected to have very similar chemical shifts.

Direct evidence for the structural arrangement in the *a-b* projection (viewing down the main channels) has been obtained by high-resolution transmission electron microscopy, as shown in Fig. 2. The schematic projection for the proposed structure (oxygen atoms omitted for clarity) is inset, together with a theoretical image, computed for specimen thickness, operational parameters and imaging defects of the microscope¹².

The proposed structure of Theta-1 is the first example of a new topologically-distinct structure-type. In accordance with IUPAC recommendations used to classify zeolites and related tectosilicates⁹, we propose to designate this new structure type as TON. The high-silica content and the 10-T-ring channels give Theta-1 aluminosilicate similar properties to other medium-pore high-silica zeolites such as ZSM-5, ZSM-11 and ferrierite. However, molecular shape selective catalysis in zeolites is highly dependent on the geometry and tortuosity of the channel and cavity systems¹³. Matching the geometries of these channels and cavities with those of the reactants, products and transition states in a catalytic reaction gives insight into the zeolite catalytic activity and selectivity. Thus, the unique high-silica unidimensional 10-T-ring channel system, particularly the lack of channel intersections and associated 'cage' volumes¹⁴, should give Theta-1 valuable structure-specific properties. The natural zeolite Laumontite¹⁰ also has a unidimensional 10-T-ring structure. However, it is a low-silica zeolite ($\text{SiO}_2:\text{Al}_2\text{O}_3$ range 3.5-4.56) and thus can be expected to have a low thermal stability in the hydrogen-form for many of the catalytic and separation applications potentially feasible for Theta-1.

Recently, several novel zeolites have been reported; Nu-10 (ref. 15), KZ-2 (ref. 16), ISI-1 (ref. 17) and ZSM-22 (ref. 18). Their X-ray powder diffraction patterns are consistent with their also having the TON structure type.

We thank B. M. Maunders, R. M. Highcock, I. D. F. Poplett, S. Ramdas, D. M. Thornley and D. Wood (BP Research Centre), G. R. Millward (University of Cambridge) and J. Dwyer (UMIST) for valuable contributions, S. Cartledge (BP Research Centre) for helpful discussions and BP International PLC for permission to publish.

Received 23 July; accepted 2 October 1984.

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The α -recoil effects of uranium in the Oklo reactor

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Until recently, it was thought that chemical elements were synthesized only in stars. The discovery of the Oklo phenomenon¹⁻⁶ in 1972 revealed, however, that there was a nuclear 'fire' on the Earth 1,700 Myr ago. We have now carried out a series of acid-leaching experiments on a sample of uranium ore from reactor zone no. 10 of the Oklo mines in Gabon, Africa, and observe anomalously high $^{234}\text{U}/^{238}\text{U}$ ratios accompanied by modestly increased $^{235}\text{U}/^{238}\text{U}$ ratios in uranium fractions. These results, which can be interpreted as being due to the α -recoil effects of ^{238}U and ^{239}Pu , provide a convenient way of calculating the conversion factor (or the fraction of uranium atoms converted to plutonium) of the natural reactors from radiochemical data, obviating the necessity for mass-spectrometric measurements.

Almost 30 years ago, Cherdynsev⁷⁻⁹ discovered that the $^{234}\text{U}/^{238}\text{U}$ ratio does not remain constant in a mineral extract: for example, uranium in a zircon extract showed a $^{234}\text{U}/^{238}\text{U}$ ratio of 1.8 (Ci/Ci) while that of surface waters was sometimes as high as 7-8. Kigoshi¹⁰ proposed that the excess ^{234}U may come from α -recoil ^{234}Th atoms ejected into the groundwater. Kobashi *et al.*^{11,12} have carried out a series of leaching experiments with the radioactive minerals euxenite, uraninite and samarskite. Their results indicate that the observed isotopic fractionations of uranium, thorium and radium can be attributed to the difference in physicochemical state between the isotopes in the minerals and can be explained in terms of: (1) damage due to α -recoil displacement; (2) annealing; and (3) the difference in oxidation states between ^{238}U and ^{234}U . Anomalously high $^{234}\text{U}/^{238}\text{U}$ ratios are sometimes found in secondary minerals containing uranium. For example, Sakanoue and Komura¹³ found a value of ~ 15 for a sample of maghaemite from the Abeshiro mine in Aomori Prefecture, Japan. Detailed studies on the nature of α -recoil and preferential solution effects have recently been carried out by Fleischer and co-workers¹⁴⁻¹⁶ using the fission track counting method. We used 1.089-g sample of powdered uranium ore from reactor zone 10 of the Oklo uranium deposit in the Republic of Gabon, Africa. The uranium content of this ore sample, which was a steel-grey colour, was 34.5%

and the $^{235}\text{U}/^{238}\text{U}$ ratio was 0.660 atom %. It was leached in a series of experiments, beginning with 40 cm³ of 0.1 M HCl, and was subsequently treated successively with 6 M HCl, 9 M HCl and 8 M HNO₃. The first passage of the 8 M HNO₃ solution through the column resulted in a marked increase in the rate of reaction. When this step was completed, the residue was transferred to a small beaker and treated with 8 M HNO₃ for ~ 3 h at near boiling point. The residue was filtered and washed with 8 M HNO₃. The little residue which remained after this was transferred to a Teflon beaker and treated with concentrated HF.

The radiochemical procedures used to separate and purify the uranium were essentially those used by Sakuragi *et al.*¹⁷. The α -spectrometry was carried out with an ORTEC-576 α -spectrometer with dual silicon surface barrier detectors (ORTEC BR-21-300-100) attached to a multiplexer/router (ORTEC-476) and a multichannel analyser (Norland Ino-Tech IT-5300). The FWHM for the ^{234}U and ^{238}U peaks is ~ 40 -50 keV. Because α energies of ^{226}Ra (4.785 MeV) and ^{230}Th (4.684 MeV) are close to that of ^{234}U (4.773 MeV), we made sure that thorium and radium had been completely removed from the purified uranium fraction.

Table 1 shows the experimental results. Figure 1 compares the α spectra of fractions 4 and 10. The most remarkable feature of our results is the extremely high $^{234}\text{U}/^{238}\text{U}$ ratio of the uranium fraction from leaching step 10 (64.3 ± 3.0), which is far greater than the values reported by previous investigators with various radioactive minerals^{9,11,12}.

Most of the uranium in the ore sample had been dissolved in the acid solutions by the time the 8 M HNO₃ treatment was completed and leachate fraction 7 was obtained. The observed $^{234}\text{U}/^{238}\text{U}$ ratios in leachates 3-7 were essentially the same as the radioactive equilibrium value of 1.00. The marked increases observed in the $^{234}\text{U}/^{238}\text{U}$ ratios in fractions 8-11 may be due to the fact that we are observing a preferential solution effect of recoil atoms of ^{234}U present in mineral phases whose uranium content was much lower than that of the reactor core material. The Oklo ore sample studied here contained tiny inclusions of haematite, clay and breccia. These inclusions, which contained very little uranium initially, may have been accumulating recoil atoms of ^{234}U over many years. The ^{234}U atoms which entered the crystal lattices of the mineral inclusions would be loosely held and could be leached by the acid solutions much more easily than any ^{235}U and ^{238}U originally present. Alternatively, if the effects are due to emplacement of recoiling nuclei into a second, relatively low-uranium phase adjacent to the dominant uranium-rich mineral, direct dissolution of the surface layers would remove the implanted atoms and so produce the observed effect.

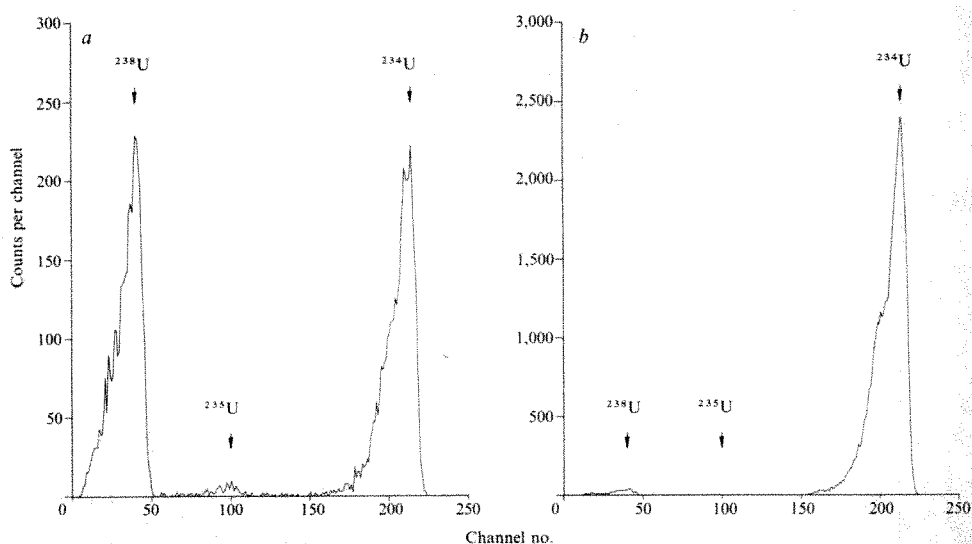


Fig. 1 The α spectra of uranium fractions: *a*, from fraction 4 obtained after treating with 200 cm³ of 6 M HCl; *b*, from fraction 10 obtained after treating with 50 cm³ of hot 8 M HNO₃.

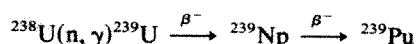
Table 1 Variation of the isotopic composition of uranium extracted from a sample of Oklo ore deposit in the acid-leaching experiments

Fraction no.	Acid used	Amount of U leached (mg)	$^{234}\text{U}/^{238}\text{U}$ (Ci/Ci)	$^{235}\text{U}/^{238}\text{U}$ (Ci/Ci)	Notes
1	0.1 M HCl*	70.6	1.15 ± 0.03	0.035 ± 0.004	Leached with 40 cm ³ of acid for 1 day
2	0.1 M HCl†		1.22 ± 0.02	0.037 ± 0.003	
3	6 M HCl*		0.97 ± 0.02	0.039 ± 0.003	
4	6 M HCl†		1.02 ± 0.03	0.036 ± 0.004	
5	9 M HCl*	91.8	1.03 ± 0.05	0.035 ± 0.006	Leached with 40 cm ³ of acid for 2-3 h
6	9 M HCl†		1.01 ± 0.03	0.036 ± 0.005	
7	8 M HNO ₃ *		1.01 ± 0.04	0.045 ± 0.007	
8	8 M HNO ₃ ‡		4.14 ± 0.28	0.051 ± 0.014	
9	8 M HNO ₃ §	12.3×10^{-3}	5.47 ± 0.42	0.065 ± 0.019	Leached with 60 cm ³ of acid for 2-3 h
10	8 M HMO ₃ (hot)		64.3 ± 3.0	0.120 ± 0.017	
11	Conc. HF (hot)		21.02 ± 0.51	0.064 ± 0.006	

The sample was packed in a plastic column of a diameter 1 cm attached to a glass container into which a certain volume of acid solution was introduced. The acid solution was allowed to flow through the column containing the uranium ore sample at a slow rate and collected in a small beaker or a test tube. Only a small volume of leachate solution, which was collected at the beginning and at the end of each leaching step, was analysed for the uranium isotopes. A pressure of ~0.5-1.5 m (water-equivalent) was applied to the leaching column to speed up the passage of the acid solutions through the ore sample.

*Initial fraction. †Final fraction. ‡Final fraction 1. §Final fraction 2.

A marked increase in the $^{234}\text{U}/^{238}\text{U}$ ratio observed in leachate fractions 8-11 is accompanied by a modest increase in the $^{235}\text{U}/^{238}\text{U}$ ratios (Table 1, Fig. 2). The observed increase in the $^{235}\text{U}/^{238}\text{U}$ ratio in some of the leachate fractions of the Oklo ore samples is to be expected, because the reactions

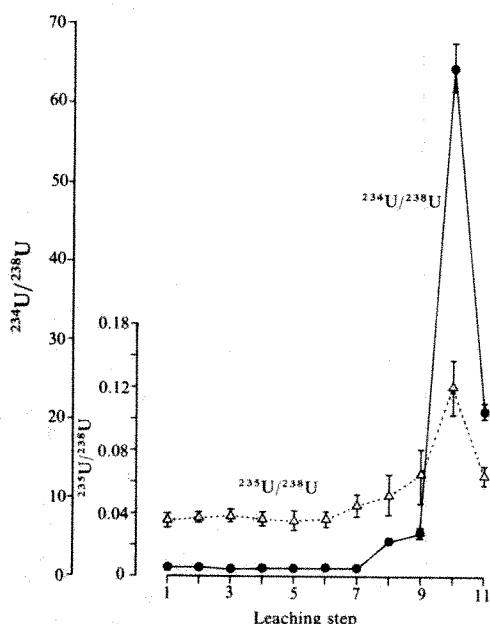


must have occurred on a large scale when the Oklo reactors were in operation. The α -recoil of the decaying ^{239}Pu atoms may have implanted the atoms of ^{235}U in the crystal lattices of nearby mineral inclusions.

The experimental data obtained here can be used to calculate the values of conversion factor C for the natural reactors, which can be defined as

$$\begin{aligned} \frac{dN_{235}}{dt} &= -N_{235}\sigma_{235}(1-C)\Phi \\ &= -N_{235}\sigma_{235}\Phi + N_{239}\lambda_{239} \end{aligned} \quad (1)$$

where Φ is the neutron flux, σ_{235} is the neutron-absorption cross-section of ^{235}U and λ_{239} is the decay constant of ^{239}Pu .

**Fig. 2** Variation of the $^{234}\text{U}/^{238}\text{U}$ and $^{235}\text{U}/^{238}\text{U}$ ratios of uranium extracted from a sample of Oklo ore deposit.

Because the same relationship holds for the ^{235}U atoms (N_{235}^P) which were created by the decay of ^{239}Pu atoms, we also have

$$\frac{dN_{235}^P}{dt} = -N_{235}^P\sigma_{235}\Phi + N_{239}\lambda_{239} \quad (2)$$

and, solving equations (1) and (2), we have

$$\frac{1}{C} = \frac{\log(N_{235}/N_0)}{\log\left[1 - \left(\frac{N_{238}}{N_{235}}\right)\left(\frac{A_{235}^P}{A_{234}}\right)\left(\frac{T_{235}}{T_{238}}\right)\right]} + 1 \quad (3)$$

where A_{235}^P is the activity of recoiled ^{235}U atoms from ^{239}Pu , A_{234} is the activity of recoiled ^{234}U atoms from ^{238}U , T_{235} and T_{238} are the half lives of ^{235}U and ^{238}U , respectively, and N_{235}/N_0 is the ratio of the ^{235}U contents of the uranium in the natural reactor and in the normal uranium ore, respectively.

For the uranium sample from reactor zone 10 of the Oklo mines, we have $(N_{235}/N_0) = 0.00660/0.00725$, $(A_{235}^P/A_{234}) = (0.120-0.036)/(64.3-1.0) = 0.084/63.3$ (see the data shown in Table 1, fraction number 10) and equation (3) yields a value of $C = 0.26$. Similarly, the observed $^{234}\text{U}/^{238}\text{U}$ and $^{235}\text{U}/^{238}\text{U}$ ratios for fraction 11 yield a value of $C = 0.27$. Hageman *et al.*¹⁸ and Neuilly *et al.*¹⁹ have reported values of C ranging from 0.37 to 0.58 and from 0.12 to 0.69 for a number of Oklo samples which they studied, respectively.

The investigation was supported by NSF grant ATM 82-06718. We thank Professor Jingui Deng of the Beijing Institute of Uranium Geology for providing the Oklo reactor sample.

Received 2 April; accepted 15 August 1984.

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A new heteropolyacid with carbon as a heteroatom in a Keggin-like structure

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In the group of compounds called heteropolyacids based on heteropolyanions represented by the general formula $(M'_xM_yO_z)^{m-}$, where the host atom M is usually tungsten or molybdenum, the most familiar are the 12-heteropolyanions (or acids) in which $x:y = 1:12$ and the heteroatom M' is Si, Ge, Sn, P, As, or the like. Structural investigations¹⁻³ have shown that the anion has a cage-like structure known as the Keggin structure. Generally, 12-heteropolyacids with this structure are strongly acidic and are also remarkably stable; $H_3(PW_{12}O_{40}) \cdot nH_2O$, for example, can be solidified from solution. I have obtained a novel compound believed to be a kind of 12-heteropolyacid (to be termed dodecatungsto-carbonic acid) with carbon as the heteroatom. The constitution of the new material is supported by the results of infrared and Raman spectroscopy.

Tungsten carbide WC (commercial powder) was dissolved in a 15% hydrogen peroxide (H_2O_2) solution, giving a strongly acidic solution almost without colour. (Although the reaction of WC with H_2O_2 appears not previously to have been reported, reactions of Mo (metal) and MoO_3 with H_2O_2 are known.⁴) Excess H_2O_2 was removed by catalytic decomposition using a platinized net, and the small amount of carbon soot floating in the solution, probably caused by free carbon in the WC, was carefully removed using 0.6- μm filter paper. The clear solutions thus obtained were dried at 50 °C and at 100 °C to give yellow non-crystalline solids (solid I and solid II, respectively).

The amount of CO_2 evolved from solid II by heating at 650 °C in an oxygen stream was measured by gas chromatography. From the weight of the residual solid, which appeared to be pure WO_3 , and the amount of CO_2 evolved, the ratio C/W in solid II was determined to be 1:12.00 (with a standard error of 0.45). No significant differences of the ratio were found when solid II was heated in an O_2 or He stream, indicating that its carbon is not in elemental form.

Thermal decomposition of solid II was followed by thermogravimetric analysis (TG) and differential thermal analysis (DTA) in a dry air stream, and solid II was found to decompose below 700 °C (Fig. 1) in two steps, each accompanied by an endothermic peak. The sole exothermic peak at 450 °C, which is not accompanied by weight change, may represent a crystal

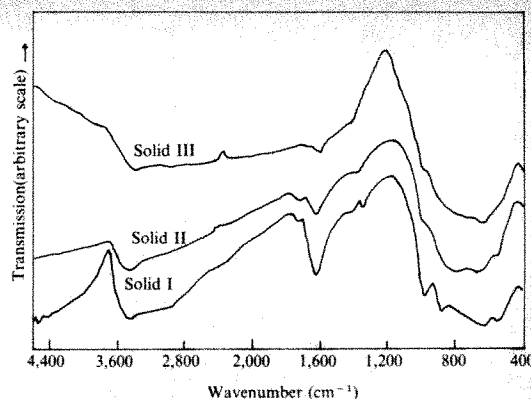


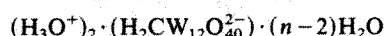
Fig. 1 Thermogravimetric (TG) and differential thermal analysis (DTA) for solid II. Initial sample weight, 72.1 mg; heating rate (T), 10 °C min⁻¹; atmosphere, dry air (30 cm³ min⁻¹).

phase change. Identical results were obtained in an Ar stream, showing that oxidation (for example, of CO) could not have been involved. The first endothermic step in the decomposition was shown to be dehydration by heat-treating solid II at 200 °C for 40 min, when CO_2 analysis of the material (solid III) revealed that the ratio C/W is again 1:12.

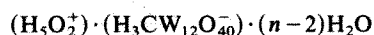
From the amount of weight loss in the second step, beginning at 280 °C, the empirical formula of the compound in the intermediate stage (solid III) is calculated to be $CO_2 \cdot 12WO_3 \cdot 4H_2O$ and, with the help of the TG data, the composition of solid II is found to be $CO_2 \cdot 12WO_3 \cdot 16H_2O$.

The infrared spectra (Fig. 2) of solids I, II and III each have a band at 1,730 cm⁻¹, although that in the spectrum of solid III is indistinct. The same vibrational frequency is characteristically found in heteropolyacids with anions of the Keggin type such as $H_3PMo_{12}O_{40} \cdot nH_2O$, and has been assigned to the frequency of pyramidal hydrated protons in groupings such as H_3O^+ (or $H_5O_2^+$) (see refs 5, 6).

These results suggest that the compound may be a type of 12-heteropolyacid with an anion whose atomic arrangement is the same as, or similar to, the Keggin structure, so that it may be written as $H_4(CW_{12}O_{40}) \cdot nH_2O$ or, more properly,



or



In an anion of Keggin-type incorporating P, for example, four W_3O_{13} units (each constructed from three WO_6 octahedra

Table 1 Vibrational frequencies of CO_3 group (cm⁻¹)

Sample	Infrared/Raman	$\nu_1(A'_1)$	$\nu_2(A''_2)$	$\nu_3(E')$	$\nu_4(E')$
Solution I	Raman	960	890	ND	640
Solid I	Infrared	970	880	1,350, 1,400	640
	Raman	970	890	ND	630
Solid II	Infrared		870	1,400	650
	Raman	950		ND	630
Solid III	Infrared		(900)	1,400	640
	Raman	950		ND	(620)
$CaCO_3^*$	Infrared		879	1,429-1,492	706
Calcite	Raman	1,087		1,432	714
$CaCO_3^*$	Infrared	1,080	866	1,492, 1,504	706, 711
Aragonite	Raman	1,084	852	1,460	704
Selection rule	D_3	Infrared	+	+	+
	C_s	Raman	+	+	+
	Infrared/Raman	+	+	+(split)	+(split)

ND, not detected; (), vague because of overlapping bands; +, active; -, inactive; *, vibrational frequencies of two types of $CaCO_3$ having different site symmetries (D_3 in calcite and C_s in aragonite) shown for comparison.

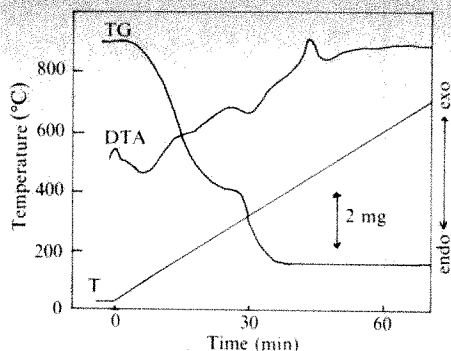


Fig. 2 Infrared spectra of solids I, II and III, recorded using the KBr pellet technique. Transmission has an arbitrary scale. Bands around 3,400 and 1,620 cm^{-1} are due to H_2O in the solid states. Kinks around 2,400 cm^{-1} arise from the influence of CO_2 in the atmosphere.

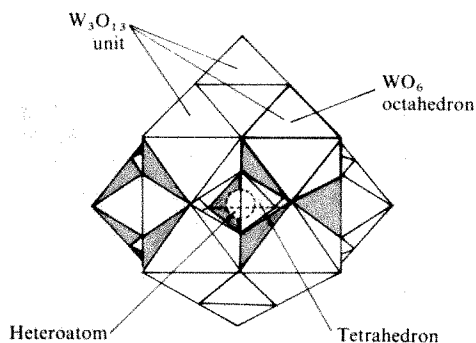


Fig. 3 Schematic structure of heteropolyanion $(\text{XW}_{12}\text{O}_{40})^{m-}$ with the Keggin structure. A heteroatom such as P is placed in the centre of the tetrahedron, whereas carbon in these compounds may occupy the centre of one of the tetrahedral surfaces.

with common edges) are linked by common corners to give a tetrahedral cavity (Fig. 3). A heteroatom such as P at the centre then provides a tetrahedral PO_4 group with four oxygens for each W_3O_{13} unit. Specific bands due to vibration of the XO_4 group can be seen in infrared and Raman spectra of compounds with this structure.

The infrared band around 1,400 cm^{-1} is, however, distinctive for the present compounds and must be assigned to one of the vibrations due to the C—O bonds (Fig. 2). Although the CO_4 group, the orthocarbonate anion, is formally equivalent to PO_4 or SiO_4 , this is an uncommon grouping for carbon which is found only exceptionally in esters such as $\text{C}(\text{OCH}_3)_4$. Moreover, the vibrational frequencies due to CO_4 are unlikely to appear in the vicinity of 1,400 cm^{-1} .

It is, therefore, more reasonable to suppose that carbon in these compounds is formed into the familiar CO_3 group. An infrared active band (or bands) due to the ν_3 of this planar group is usually found in the range 1,400–1,500 cm^{-1} as, for example, in calcite⁷. From among the vibrational frequencies observed in the infrared and Raman spectra, those which can be assigned to the fundamentals of CO_3 are shown in Table 1. Other bands below 1,000 cm^{-1} are attributed to the WO_6 groups.

The symmetry (D_{3h}) of CO_3 in an isolated state is usually lowered in both the solid state and in solution by interactions with ligands or surrounding molecules, resulting in modified selection rules and/or the splitting of degenerate bands. The two infrared bands at 1,350 and 1,400 cm^{-1} for solid I are due to the splitting of ν_3 at sites of C_s symmetry, but the corresponding frequency is not split in solids II and III, indicating D_3 symmetry. The Raman bands in solids I and II at 950–960 cm^{-1} , which are due to the totally symmetric vibration (ν_1), are Raman-active only in D_3 symmetry and cannot be detected in the infrared spectra of my samples. The frequencies ν_2 of solids II

and III are seen at 880–890 cm^{-1} only in the infrared spectra, in agreement with the selection rule. In the case of C_s symmetry, all four vibrations must be active in both infrared and Raman modes, and these bands are indeed found in the spectra of solid I except for the Raman band ν_3 , which was presumably not detected because the signals are too weak.

These observations are evidence for the existence of a novel heteropolyacid, $\text{H}_4(\text{CW}_{12}\text{O}_{40}) \cdot n\text{H}_2\text{O}$, based on the anion $(\text{CW}_{12}\text{O}_{40})^{4-}$ (or, more properly, $(\text{H}_2\text{CW}_{12}\text{O}_{40})^{2-}$ or $(\text{H}_3\text{CW}_{12}\text{O}_{40})^-$) which incorporates the CO_3 group. This may be a kind of carbonato-complex in which carbon occupies the centre of one surface of the tetrahedron in the Keggin structure, to give rise to bonds with three oxygens out of four belonging to each W_3O_{13} ligand.

I thank Drs M. Katsumoto, J. Shimada and T. Iwayanagi for helpful discussions.

Received 24 April; accepted 24 May 1984.

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Major ion chemistry of the Ganga–Brahmaputra river systems, India

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Rivers are the main pathways by which continental weathering products are transported to the oceans so that the chemistry of river waters and the flux of elements transported by them to the oceans are central to our understanding of the exogenic cycles of elements. There have been several detailed studies of major rivers^{1–5} and we now report the major ion chemistry of another, the Ganga–Brahmaputra. These are two of the large rivers which drain the Indian subcontinent and they transport annually $\sim 10^{15}$ l of water and $\sim 2 \times 10^{15}$ g suspended matter to the Bay of Bengal^{6,7}. On a global scale, these rivers rank fourth in terms of flow, and first in terms of sediment transport^{6,7}. Studies on the continental denudation rates and the budgets of major elements in the ocean, would, therefore, be incomplete without good geochemical data on these rivers. Our studies show that the Ganga–Brahmaputra system transports some 118 million tons of dissolved solids annually to the Bay of Bengal and that their water chemistry is dictated by the weathering of carbonates and contributions from soil salts and/or saline groundwaters.

The Ganga originates in the Gangotri glacier in the Kumaun Himalayas and is known as the 'Bhagirathi' at its source. The Alaknanda is the major tributary of the Bhagirathi, and after their confluence at Devprayag the river acquires the name 'Ganga' (Fig. 1). The major tributaries of the Ganga are the Gomti, Ghaghara and Gandak from the north and the Yamuna and Son from the south. The Chambal, Betwa and Ken are the major tributaries of the Yamuna. Although all these rivers are perennial, the peak flow in all of them occurs during the south-west monsoon, July–September. The Brahmaputra originates in the Chamyungdung glacier in the Tibetan Himalayas. Several tributaries join the main river in the Assam valley, a major one being the Manas. The Ganga and the Brahmaputra merge in Bangladesh, before their outfall into the Bay of Bengal.

The Ganga and its tributaries were sampled during three seasons, March, September and December 1982, representing

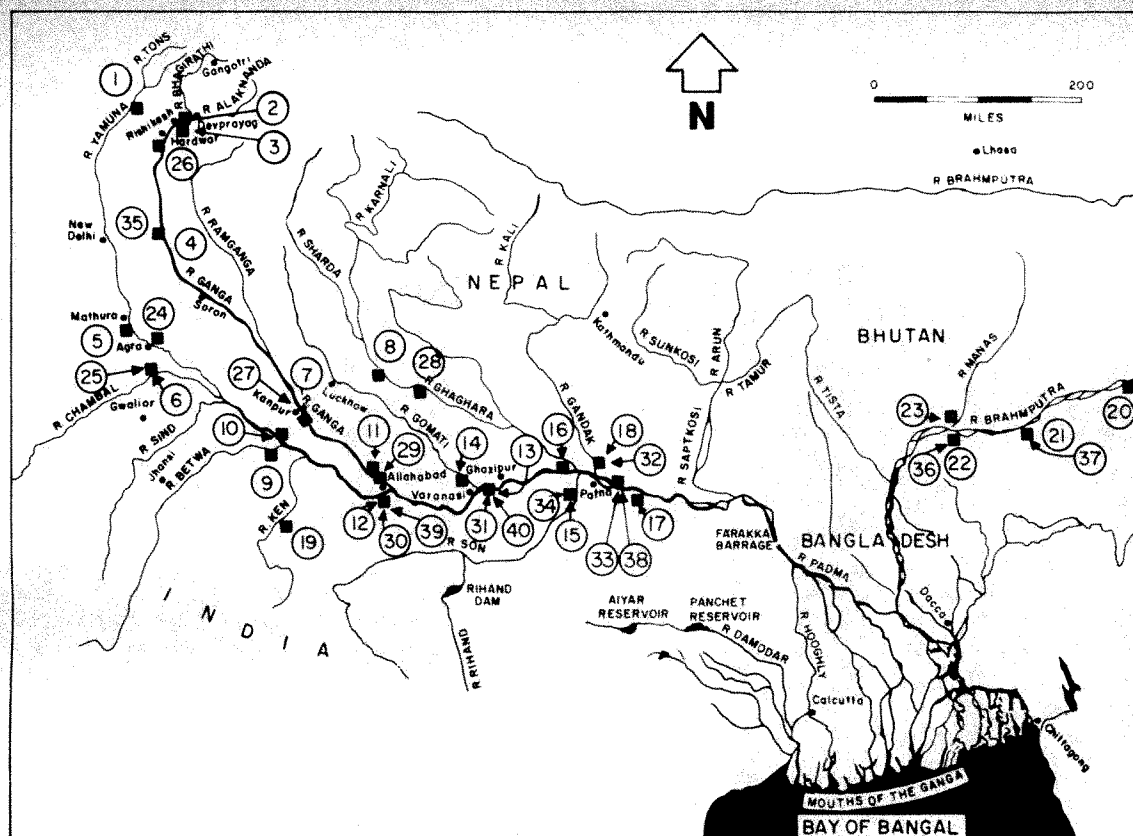


Fig. 1 Sample location map. Numbers correspond to sampling sites in March–April (1–23); September (24–35) and December (36–40) 1982.

Table 1 Major ion composition ($\mu\text{mol l}^{-1}$) of the Ganga and Brahmaputra rivers and their tributaries

Sample code*	Location	Na	K	Mg	Ca	HCO ₃	Cl	SO ₄	SiO ₂	TDS (mg l ⁻¹)
Highland rivers										
26, Ganga	Hardwar	59	38	153	353	894	23	121	109	94
28, Ghaghara	Ayodhya	96	59	232	528	1,601	26	76	114	144
32, Gandak	Hajipur	74	84	259	649	1,860	32	103	143	170
22, Brahmaputra	Goalpara	80	46	148	318	884	29	99	118	92
23, Manas	Goalpara	94	42	181	348	930	26	113	131	99
Lowland rivers										
24, Yamuna	Agra	1,401	130	395	794	1,921	1,172	412	170	287
30, Yamuna	Allahabad	556	57	190	547	1,753	222	67	164	173
25, Chambal	Dholpur	593	53	337	829	2,531	284	76	296	247
34, Son	Ara	240	40	166	434	1,372	71	22	210	129
Ganga main channel										
26, Ganga	Hardwar	59	38	153	353	894	23	121	109	94
35, Ganga	Garhmukteshwar	105	68	182	471	1,159	19	143	127	121
27, Ganga	Kanpur	354	82	242	565	1,829	78	103	157	173
29, Ganga	Allahabad	425	79	201	458	1,570	116	85	121	151
31, Ganga	Varanasi	473	62	188	514	1,707	162	72	147	164
33, Ganga	Patna	280	63	203	547	1,707	87	58	129	156

Measurements were taken during peak flow for all rivers (September 1982) except for the Brahmaputra and Manas, for which measurements were taken during moderate flow (April 1982).

* Numbers correspond to locations in Fig. 1.

respectively lean, peak and moderate flow conditions. Samples from the Brahmaputra and Manas were collected during April and December 1982 (see Fig. 1 for the sampling sites). All the samples were taken from surface waters, collected from the mid-channel of the river either from highway bridges or by going into the rivers using small boats. Details of sampling and their pretreatment are discussed elsewhere⁸. pH, dissolved O₂, conductance and bicarbonate were measured on site. An aliquot of the water sample was filtered within ~6 h of collection through a 0.45- μm Millipore filter using a polycarbonate filter system⁸.

The filtered water was split into two fractions, one of which was preserved unacidified and the other acidified to pH ~2 using HNO₃. The filtered unacidified samples were analysed in the laboratory for major cations and anions using appropriate methods⁸. The major ion composition of the Ganga and its tributaries during their peak flow (samples 24–35; Fig. 1) and that of the Brahmaputra and Manas during their moderate flow (samples 22 and 23; Fig. 1) are given in Table 1. The 'highland rivers' cover the Ganga (up to Hardwar), Ghaghara, Gandak, Brahmaputra and Manas, the 'lowland rivers' the Yamuna (at

Table 2 Average major ion composition ($\mu\text{mol l}^{-1}$) of the Ganga and Brahmaputra rivers and their tributaries

River	Na	K	Mg	Ca	HCO ₃	Cl	SO ₄	SiO ₂	TDS (mg l ⁻¹)
Ganga*	533	83	257	475	1,751	146	112	112	170
Ghaghara	131	61	270	562	1,777	29	82	124	159
Gandak	104	85	285	655	1,886	52	130	147	177
Yamuna†	933	62	290	573	2,183	382	120	157	222
Chambal	827	56	374	792	2,627	337	120	260	262
Betwa	2,377	53	690	502	4,238	426	71	237	388
Ken	701	54	461	550	2,683	233	52	214	241
Son	277	43	191	464	1,507	93	27	214	142
Manas	94	42	181	348	930	26	113	131	99
Brahmaputra	92	48	157	351	957	31	106	130	100
Ganga‡	419	67	269	583	1,966	140	83	132	183
World averages§	191	38	166	398	1,019	105	96	207	115

See text for calculations of the average composition.

* At Allahabad, before confluence with the Yamuna; † At Allahabad, before confluence with the Ganga; ‡ at Patna, after confluence of the Ghaghara, Son and Gandak; § from ref. 4.

Allahabad), Chambal and Son. For brevity, the results from other seasons are not given here, but they are used to derive the average chemical composition of these river waters (Table 2).

The major ions in river waters are supplied from the atmosphere and from the weathering of rocks/soils in the drainage basin. The atmospheric contribution consists of two components, the marine cycle salts and the terrestrial inputs. Our study did not include the chemical analysis of rain water and thus it is difficult to obtain a precise estimate of the atmospheric supply of major ions to the river waters during the sampling period. However, the atmospheric contribution of marine cycle salts has been estimated from the available rain water data⁹, assuming that the measured chloride concentration in the highland rivers, during their peak flow, is solely due to the marine contribution⁸. These calculations suggest that, during peak flow, about 30% of sodium in the highland rivers is of marine origin⁸. Of the lowland rivers, the Son has the largest marine component, 45% Cl and 11% Na. The marine contribution of other major ions to these rivers is negligible. The estimated marine cycle salt contribution is an upper limit in that we have assumed that no chloride is supplied to the river water by weathering processes. That contribution can be taken to be small from the observation that the Cl concentration in highland rivers, during their peak flow (23–32 $\mu\text{mol l}^{-1}$, Table 1), is very similar to that in rain water from inland stations (18–41 $\mu\text{mol l}^{-1}$, ref. 8).

In the highland rivers, Ca, Mg and HCO₃ are the most abundant dissolved species in all the seasons sampled. The cations Ca and Mg account for 84–92% of total cations (on an equivalent basis); the (Ca + Mg): (Na + K) ratio ranges between 5.2 and 11.5. HCO₃ constitutes 73–91% of the anions, sulphate accounting for most of the remainder (Tables 1, 2). These abundances favour carbonate weathering as source, which is

consistent with the regional lithology of the rivers, whose upper reaches are composed of Siwalik sediments and the limestones, dolomites and calcareous shales of the Deoban-Tejam zone^{10,11}. The sulphate in the waters may originate from the weathering of gypsum/anhydrite or by the oxidation of pyrite; in the Kumaun Himalayas, gypsum, has been reported as a common accessory mineral¹⁰ and in the Brahmaputra basin, pyritic sediments are present^{4,11}. Furthermore, the low abundance of silica and the high (Ca + Mg): (Na + K) ratios suggest that the contribution of major ions to these waters by silicate weathering has only minor significance.

In the Yamuna (at Allahabad), and the Chambal and Son during peak flow, (Ca + Mg) accounts for 71–81% of the total cations and HCO₃ accounts for 83–92% of the anions (Table 1). As in the highland rivers, the dominance of Ca, Mg and HCO₃ in the major ion composition is attributable to carbonate weathering. The Chambal drains the Vindhyan sandstones, quartzites, siliceous limestones and dolomites, whereas the stretch of the Yamuna between Saharanpur and Allahabad consists of alluvium rich in 'Kankar' carbonates¹². These three rivers were also sampled during the lean flow season. The water chemistry of the Son shows no significant variation with the seasons. In contrast, in the Yamuna and the Chambal, the major ion ratios differ significantly with the flow conditions⁸. During lean flow, the concentrations of Na, Cl and SO₄ increase considerably and account for nearly a third of the major ions, the alkaline and saline soil salts in the drainage basins and/or effluent seepage of saline groundwaters. The other lowland rivers, the Gomti, Betwa and Ken, were sampled only during lean flow (Table 2). The water chemistries of the Son and Ken are similar. During lean flow, the Betwa waters are very saline and are second only to the Yamuna in terms of the abundance of total dissolved salts (TDS).

Table 3 Average annual fluxes (10^9 mol yr^{-1}) of dissolved species through Ganga-Brahmaputra river system

River	Discharge ($10^{12} \text{ l yr}^{-1}$)	Na	K	Mg	Ca	HCO ₃	Cl	SO ₄	SiO ₂	TDS (M ton yr ⁻¹)
Ganga*	59	31	4.9	15	28	103	8.6	6.6	6.6	10
Yamuna	93	87	5.8	27	53	203	36	11	15	21
Chambal	30	25	1.7	11	24	79	10	3.6	7.8	7.8
Ghaghara	94	12	5.8	25	53	168	2.7	7.8	12	15
Son	32	8.9	1.4	6.1	15	48	3	0.9	6.9	4.5
Gandak	52	5.4	4.4	15	34	98	2.7	6.8	7.7	9.2
Ganga†	364	153	24	98	212	716	51	30	48	67
Brahmaputra‡	510	47	24	80	179	488	16	54	66	51
World rivers§	31,400	6,000	1,200	5,200	12,500		3,300	3,000	6,500	3,600

Discharge data from ref. 18.

* At Allahabad before confluence with Yamuna; † At Patna after confluence with Ghaghara, Son and Gandak; ‡ at Goalpara; § from ref. 4.

The downstream and seasonal variations in the major ion chemistry of the Ganga main channel (Table 1) are controlled by the chemistry of its tributaries and their mixing volumes. The $(Ca + Mg) : (Na + K)$ equivalent ratio decreases downstream, but more markedly during lean flow^{8,14}, when the ratio becomes as low as 1.25 at Varanasi, after the confluence of the Yamuna. The relative increase of $(Na + K)$ during lean flow is attributable to supply from soil salts in the alluvial plains and/or effluent seepage from saline groundwaters. The TDS of the Ganga main channel, during peak flow, range between 94 and 173 mg l⁻¹ between Hardwar and Patna (Table 1). The maximum TDS concentration, 401 mg l⁻¹, was observed during lean flow at Varanasi. The TDS concentration and range that we observed in the Ganga main channel, during peak flow, is about a factor of three lower than that reported¹⁵ (227–502 mg l⁻¹) during the wet monsoon in 1977.

The average major ion compositions of the rivers sampled are given in Table 2. For the Ganga and its tributaries, the average concentration, $\bar{C}$, was calculated as $\bar{C} = 0.83 C_p + 0.17 C_l$ where C_p and C_l are the concentration of the species during peak and lean flow conditions. The factors 0.83 and 0.17 are based on the monthly discharge data for the Ganga at Farakka¹⁶, which show that 83% of water transport occurs during July–September and the remaining 17% during the rest of the year. The major ion abundances, measured in September and March 1982, are taken to be typical of peak and lean flow conditions. The discharge data for the Brahmaputra at Gauhati¹⁷ show that moderate/peak flow conditions occur during April–October and that 86% of water discharge is during this period. For the Brahmaputra, $\bar{C} = 0.86 C$ (April) + 0.14 C (December). For the Betwa, Ken and Manas, the measurements were taken during one season only. Although these results are presented as their average composition (Table 2), they are not used for flux calculations.

A comparison of the average chemical composition and the TDS content of the Ganga (at Patna) and the Brahmaputra (at Goalpara) shows that the Ganga waters are nearly twice as saline as those of Brahmaputra (Table 2). Although the water flux of the Ganga is ~70% of that of the Brahmaputra, the annual flux of dissolved salts carried by the Ganga (at Patna), 67 million tons, is ~30% more than that of the Brahmaputra at Goalpara. The Ganga transports nearly three times more Na and Cl than the Brahmaputra. The Na and Cl to the Ganga comes mainly from its tributaries, the Yamuna and the Chambal (Tables 1, 3).

On a global scale, the average TDS content of the Ganga and its tributaries is higher than that of the global mean river water composition (Table 2). The average TDS content of the Ganga (at Patna) is very similar to that of the Chiang Jiang of China⁴ and about five times more than that of the Zaire and Amazon⁴. The Ganga accounts for ~2.5% of the global flux of Na to the oceans (Table 3). The Ganga (at Patna) and the Brahmaputra (at Goalpara) together supply ~118 million tons yr⁻¹ of dissolved solids to the Bay of Bengal. This accounts for ~3% of the global supply of dissolved salts to the oceans, which is nearly the same as their contribution to the global water discharge.

We thank Drs B. L. K. Somayajulu and W. S. Moore for discussions, and J. P. Bhavsar, N. Hussain, W. S. Moore, P. Sharma and B. L. K. Somayajulu for much help during sampling expeditions. Financial support for this study was provided in part by grants from the PL-480 and the Department of Science and Technology to the Physical Research Laboratory, and by NSF grants INT-8117218 and INT-8218484 to Dr W. S. Moore.

Received 26 July; accepted 8 October 1984.

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Complexity of human T-cell antigen receptor β -chain constant- and variable-region genes

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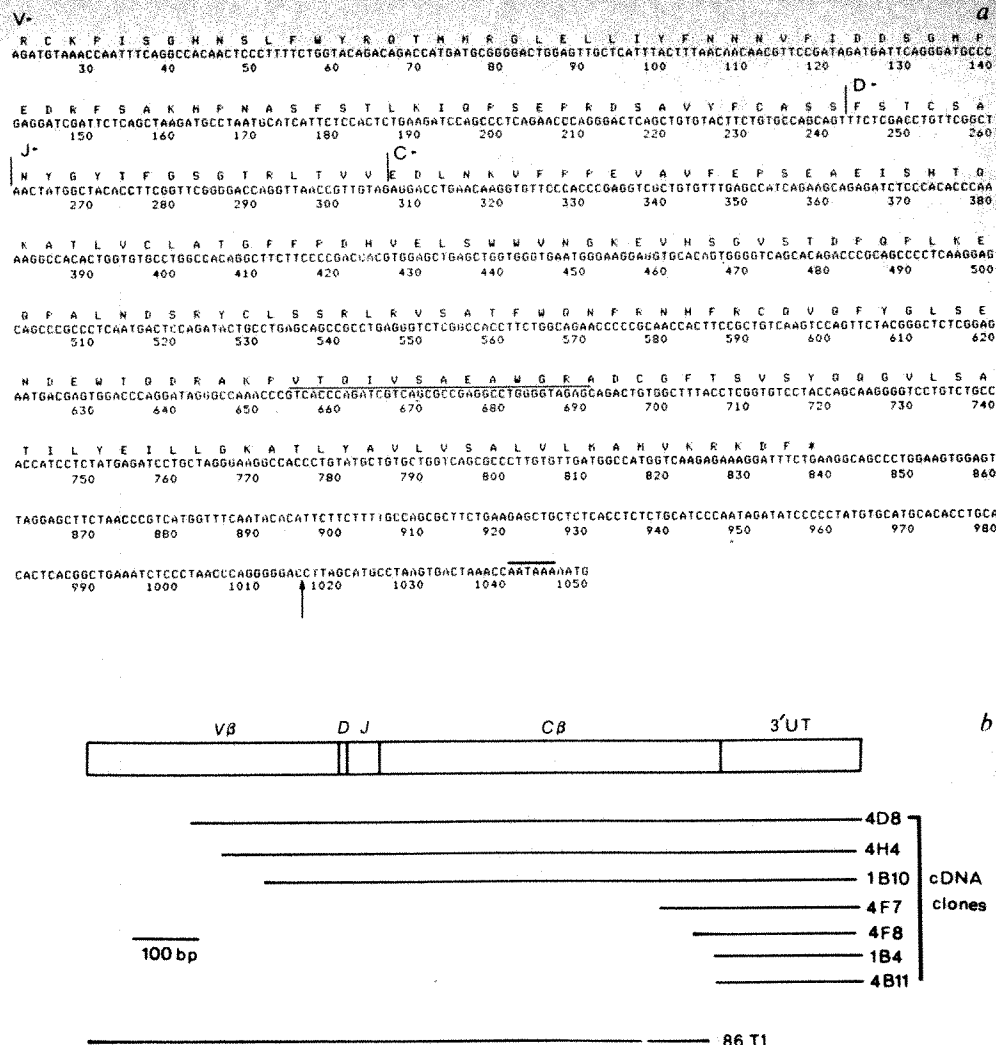
Immune systems of vertebrates function via two types of effector cells, B and T cells, which are capable of antigen-specific recognition. The immunoglobulins, which serve as antigen receptors on B cells, have been well characterized with respect to gene structure¹, unlike the T-cell receptors. Recently, cDNA clones thought to correspond to the β -chain locus^{2–4} of the human and mouse T-cell receptor have been described. The presumptive β -chain clones detect gene rearrangement specifically in T-cell DNA and show homology with immunoglobulin light chains. The similarity of the T-cell β -chain gene system to the immunoglobulin genes has been further demonstrated by the recent observation of variable- and constant-region gene segments as well as joining segments and putative diversity segments^{5,6}. We report here the characterization of cDNA and genomic clones encoding human T-cell receptor β -chain genes. There are two constant-region genes ($C_{\beta 1}$ and $C_{\beta 2}$), each capable of rearrangement and expression as RNA. The gene arrangement, analogous to that of mouse β -chain genes^{6–8}, shows strong evolutionary conservation of the dual C_{β} gene system in these two species.

The JM cell line, positive for the surface makers T1, T3, T4, T6 and T8 (ref. 9 and unpublished data), was derived from a human acute lymphoblastic T-cell leukaemia patient¹⁰. A cDNA library of 500 clones was prepared from JM RNA after removing sequences present in the cDNA that were homologous to B-cell mRNA (ref. 3; Fig. 1 legend). Clones encoding T-cell receptor β -chain sequences were isolated from this library in two related experiments. First, we screened a subset of 188 JM cDNA clones with the isolated insert from the mouse β -chain clone 86T1 (ref. 3) and found four hybridizing colonies (pJMT1B10, 4D8, 4F7 and 4H4). Independently, we had been characterizing individual clones in the library by using them as probes on Northern blots containing T- and B-cell RNA. When the isolated insert from one of these T-cell-specific clones (clone pJMT1B4) identified in this way was used to screen the same subset of 188 clones, it hybridized to the same four clones identified by the mouse β -chain probe, as well as to another two clones (pJMT4B11 and 4F8). (Clones 1B4, 4B11 and 4F8 fail to hybridize with 86T1 because they do not overlap with it; see Fig. 1.) We conclude that this group of seven clones is probably derived from the β -chain mRNA synthesized in the JM cell line.

The nucleotide sequence of the presumptive β -chain cDNA clones and the derived amino-acid sequence (Fig. 1) show that the clones derive from the homologous human T-cell receptor β -chain, as predicted from their hybridization with the mouse β -chain probe. The 3' portion of the sequence encodes the constant (C)-region segment (C_{β}) extending from nucleotide 306 to the termination codon at residue 837, and continues as a presumptive 3'-untranslated sequence. Other sections of the sequence correspond to a joining (J) sequence (nucleotides

Fig. 1 Nucleotide sequence of the β -chain cDNA clones isolated from JM RNA. **a**, DNA sequence: the positions of V, D, J and C regions are indicated, with the predicted amino acid sequence. The first codon (specifying arginine) is equivalent to codon 41 of the previously published YT35 sequence² and the complete sequence is identical with YT35 except for an extra T residue at JM position 884 in the 3'-untranslated sequence. The vertical arrow shows the position of the last residue of the YT35 sequence. The sequence AATAAA, characteristic of poly(A) addition sites¹¹, is overlined. The sequence in the coding region indicated by a line corresponds to the region used for orientation in Fig. 3. **b**, Organization of the β -chain mRNA, with the extent of the various cDNA clones covering this mRNA. DNA sequencing of the β -chain cDNA clones was carried out in M13 vectors by shotgun cloning and sequencing of *Bgl*II, *Taq*I, *Sau*3A and *Eco*RI plus *Bam*HI digests of pJMT-4D8 using dideoxy chain termination procedures¹⁵⁻¹⁹.

Methods: cDNA clones were prepared from RNA isolated essentially as described²⁰. Single-stranded cDNA was synthesized on methyl mercuric hydroxide-denatured JM poly(A)⁺ RNA and the template subsequently hydrolysed using recommended conditions²¹. The mixture was passed over a Sepharose CL-4B column in 50 mM NaOH, 1 mM EDTA, and fractions containing cDNA longer than ~200 nucleotides were precipitated with ethanol and transfer RNA carrier and resuspended in H₂O. cDNA (3 μ g) was hybridized with 300 μ g of B-cell RNA (from the Burkitt's lymphoma CA46)²² (estimated to be about a 15-fold molar excess of RNA to cDNA, based on average sizes of 700 and 2,000 nucleotides for cDNA and RNA, respectively, and the % rRNA contaminating the poly(A)⁺ RNA) in 360 μ l of 0.2 M NaPO₄, pH 6.8, 1 mM EDTA, for 16 h at 70 °C (to a R_{0t} actual of 150). The mixture was diluted with 30 vol. of 0.02 M NaPO₄, pH 6.8, bound to hydroxyapatite at room temperature, and single-stranded cDNA then eluted from the resin with 0.12 M NaPO₄, pH 6.8 at 60 °C. After rebinding and re-eluting this material, 10.6% of the total cDNA behaved as if it were single-stranded. Thus, the hybridization procedure should have resulted in approximately a 10-fold enrichment for T cell-specific sequences, assuming a 2% difference in the complexity of T- and B-cell RNA populations²³. After removal of NaPO₄ by gel filtration on Sephadex G-100, the cDNA was made double-stranded, treated with S₁ nuclease, size-selected again on Sepharose CL-4B, repaired with the Klenow fragment of DNA polymerase I, and ligated into *Sma*I-cleaved and phosphatase-treated pUC8 (ref. 24) essentially as described elsewhere²¹. Ampicillin-resistant colonies were selected after transformation into competent²⁵ LE392 (ref. 21). The yield was ~10⁶ colonies per μ g double-stranded cDNA. Approximately 5,000 colonies were screened with ³²P-labelled cDNA probes made from total poly(A)⁺ RNA of JM or CA46 cells. About 500 which apparently hybridized to the T-cell and not the B-cell cDNA probe were picked, and these comprise the T cell-enriched library referred to in this paper.



216-305), to a small diversity (*D*) sequence (nucleotides 243-260), and the remainder to a part of a variable (*V*) region segment². The *J* segment present in the JM clones is identical in sequence to the germ-line segment *J_β1-2* (ref. 5). Furthermore, the entire sequence, including the presumptive *V*, *D* and *J* segments, is identical with a β -chain sequence reported to derive from the human T-cell line MOLT-3 (ref. 2), except for one base. In addition, our sequence extends for another 36 residues at the 3' end, including the sequence AATAAA, probably representing the poly(A) addition signal in this mRNA¹¹.

The identity between the sequences of the β -chain cDNA clone derived from JM cells and a clone derived supposedly from the MOLT-3 cell line² is surprising. JM and MOLT-3 were isolated from different T-cell leukaemias and it is unlikely that identical *V-D-J* joining would have occurred independently in these cell lines. We have thus investigated the expression of *V_β* sequences in mRNA from six different human leukaemic cell lines, including JM and MOLT-3 with *V_β* and *C_β* probes

(Fig. 2). All six T-cell leukaemias express an RNA of ~1.35 kilobases (kb) which hybridizes with the *C_β* probe, showing that they all transcribe a *C_β* gene. However, only JM RNA hybridized to the *V_β* probe derived from the JM cDNA clones. Thus, although MOLT-3 cells express the *C_β* gene, they do not express a *V_β* segment homologous to the JM sequence. This conclusion is supported by Southern hybridization data. We have shown that three independent sources of JM cellular DNA, as well as J6 (a subclone of the JM cell line) DNA, contain identical *C_β* rearrangements, which are different from those present in two independent sources of MOLT-3 DNA. The identity of β -chain cDNA clones from JM (reported here) and another source of MOLT-3 (ref. 2) can therefore be attributed to trivial, not physiologically significant, causes.

The *C_β* probe hybridizes to a second, smaller species in some of the T-cell RNA preparations (Fig. 2). The origin of this RNA is unknown, although as the *V_β* probe detects only the larger of the two transcripts, the smaller may represent sterile *C_β*

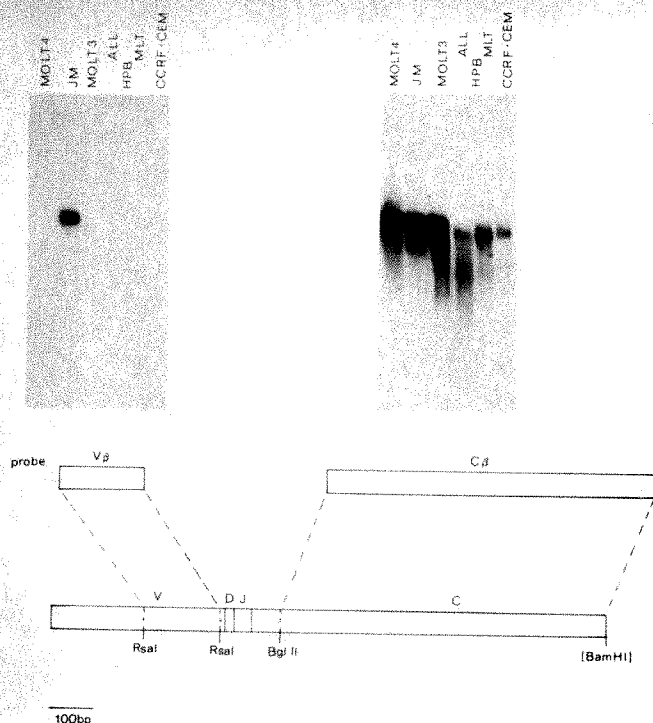


Fig. 2 Northern filter hybridization of V_{β} - and C_{β} -specific probes with RNA prepared from various T-cell leukaemia lines. Duplicate Northern filters were prepared and hybridized essentially as described²⁶ with either 1 μ g poly(A)⁺ RNA (in the JM and MOLT-4 lanes) or 20 μ g total RNA (in the other lanes) fractionated on 1.5% agarose gels. One filter was probed with pUCV_{JM} (V_{β}) and the other with M13B10BB1 (C_{β}). Note that the *Bam*HI site at the 3' end of the diagram of the C_{β} cDNA insert is from the pUC vector polylinker site. V and C region-specific probes were prepared by subcloning from pJMT4D8. The C_{β} probe was an M13 clone extending from the *Bgl*II site, just within the C_{β} sequence (nucleotide 364 of fig. 1) to the *Bam*HI site located in the pUC vector polylinker segment just beyond the 3' end of the insert. The V_{JM} probe was a clone containing the 165-base pair (bp) *Rsa*I fragment (nucleotides 59–224 of Fig. 1), cloned into the *Sma*I site of pUC9. The cell lines MOLT-4 and MOLT-3 (ref. 9), CCRF-CEM²⁷, HPB-ALL and HPB-MLT, are from T-cell leukaemias.

transcripts lacking V segments, analogous to those sometimes made from the immunoglobulin C_{μ} gene in T cells¹².

The genomic arrangement of the C_{β} genes was studied by isolating clones from a human B-cell genomic λ phage library¹³ screened with the mouse β -chain clone, 86T1. Two overlapping clones were obtained, λ B3 and λ B17 (Fig. 3). Hybridization of these clones with a human C_{β} probe indicated the presence of two separate C_{β} genes about 10 kb apart. One gene contains a site for the restriction enzyme *Hind*III (see Fig. 3); as there is no *Hind*III site in the cDNA clone of the JM β -chain, we thought that this site might occur within an intervening sequence of the gene. We therefore attempted to orient this C_{β} gene by nucleotide sequencing from the *Hind*III site. λ B3 was digested with *Hind*III together with *Eco*RI and the resulting fragments cloned into M13mp18. Clones carrying the small *Hind*III–*Eco*RI fragment were sequenced from the *Hind*III site towards the *Eco*RI site (Fig. 3). The 237 nucleotides adjacent to the *Hind*III site were not recognizable as C_{β} gene sequence but after this point, the sequence matches a part of the C_{β} segment found in the JM C_{β} cDNA clone (underlined in the cDNA sequence of Fig. 1). A typical RNA splicing signal sequence at the junction of this region probably represents an intron/exon boundary conserved in evolution; one of the mouse C_{β} genes also contains an intron at exactly the same position^{7,8}. Comparison of the genomic and cDNA sequences indicates that the gene containing the *Hind*III site is transcribed from left to right on the map of Fig. 3. Further, other sequencing and restriction

mapping data show that this C_{β} gene differs from that present in the JM cDNA clones, and that the two C_{β} genes are transcribed in the same direction (A.T. and T.H.R., manuscript in preparation). These genes are designated $C_{\beta}1$ and $C_{\beta}2$ (see Fig. 3) by analogy with the two C_{β} genes in mouse^{7,8}.

The existence of two C_{β} genes was confirmed by Southern filter hybridization experiments. For example, digestion of DNA from the B-cell leukaemia D-PLL with *Eco*RI revealed two hybridizing fragments (11.5 and 4 kb; Fig. 4). (D-PLL has been shown to contain a germ-line C_{β} gene pattern; unpublished results.) We know from the λ clones that the $C_{\beta}2$ gene occurs in the 4-kb *Eco*RI fragment, therefore the 11.5-kb germ-line *Eco*RI fragment must contain the $C_{\beta}1$ gene. We also failed to detect any closely related C_{β} genes other than those present in the genomic λ clones.

Several different patterns of DNA rearrangement can occur in T-cell leukaemias (Fig. 4). For example, MOLT-3 DNA has both $C_{\beta}2$ genes rearranged. When MOLT-3 genomic DNA was digested with *Eco*RI and hybridized with the C_{β} probe, we found a single hybridizing band of 4 kb corresponding to $C_{\beta}2$. There was no evidence of any rearranged band or of the 11.5-kb $C_{\beta}1$ germ-line band. Therefore, MOLT-3 apparently does not contain any remnant of the $C_{\beta}1$ gene; thus it has probably been deleted from the relevant chromosomes (assuming no chromosome loss), presumably by V-gene rearrangement to the $C_{\beta}2$ gene. This interpretation was confirmed by *Sac*I digestion of MOLT-3 DNA, again revealing only the unrearranged $C_{\beta}2$ gene fragment (5.4 kb). Digestion of MOLT-3 DNA with *Hind*III, however, does show rearrangement within the $C_{\beta}2$ gene region (Fig. 4). The $C_{\beta}2$ gene overlaps two *Hind*III fragments (8.5 and 7 kb long) in unrearranged DNA such as D-PLL (Fig. 4). The 7-kb fragment, which contains the 3' end of the $C_{\beta}2$ gene, is present in MOLT-3 DNA, whereas the 8.5-kb band, containing the 5' end of the $C_{\beta}2$ gene, is absent (Fig. 4) and two new fragments (26 and 19 kb) are present, indicating rearrangement of the $C_{\beta}2$ gene on both chromosomes in MOLT-3.

An example of a single C_{β} gene rearrangement is shown by JM DNA, which shows 11.5- and 4-kb germ-line C_{β} fragments, and a new, rearranged fragment of 9.5 kb after *Eco*RI digestion. As the $C_{\beta}1$ gene is transcribed in JM, this new band must represent the rearranged $C_{\beta}1$ allele. The other $C_{\beta}1$ allele is apparently not rearranged. If gene deletion is required for $C_{\beta}2$ joining, the existence of this unrearranged $C_{\beta}1$ allele implies that the second chromosome in JM has the germ-line configuration of C_{β} genes. It is unlikely that the different fragment sizes observed are due to polymorphism in the human population as studies of six non-T-cell DNAs failed to detect restriction site polymorphisms with the enzymes used here.

In all T-cell DNAs examined so far (JM and MOLT-3 DNAs plus DNA from 10 fresh leukaemic samples), we have never observed an altered *Eco*RI or *Sac*I pattern around the $C_{\beta}2$ gene. Rearrangements at $C_{\beta}2$ can be detected, however, using *Hind*III. This implies, by analogy with the mouse C_{β} gene system^{7,8}, that each human C_{β} gene has its own joining (J) cluster. The J cluster associated with $C_{\beta}2$ lies outside both the 4-kb *Eco*RI and 5.4-kb *Sac*I fragments but within the 8.5-kb *Hind*III fragment.

Significant variability exists at the level of V_{β} sequences, as the JM V_{β} sequence hybridizes only to RNA from this cell line (Fig. 2). To investigate this further, we studied the hybridization pattern of the V_{β} probe with genomic DNA. Southern filter hybridizations using our V_{β} probe (pUCV_{JM}) reveal six major hybridizing bands in JM DNA (Fig. 4). MOLT-3 DNA contains the same bands with differing hybridization intensities, as well as three faintly hybridizing elements at ~3 kb which are absent from the JM DNA. (In similar experiments, a V_{β} probe related to that used here hybridized to four bands in genomic DNA⁵.) The complexity of the V_{β} hybridization pattern is comparable with that seen in human immunoglobulin V-gene probes¹⁴, suggesting a significant germ-line contribution to V_{β} diversity.

Our results show that the human T-cell receptor β -chain locus consists of two C genes, separated by ~10 kb of DNA, and

Fig. 3 Organization of the human C_β locus. **a**, The top line shows a restriction map of the C_β locus derived from the two indicated overlapping λ phage clones ($\lambda\beta 3$ and $\lambda\beta 17$). The most 5' $EcoRI$ site (dotted line) is absent from the phage clones and was determined by genomic hybridizations. The location of the $C_\beta 1$ gene was determined by digestion of $\lambda\beta 3$ with $EcoRI$ and $BglII$ or with $HindIII$ and $BglII$ ($BglII$ cuts within the $C_\beta 1$ coding sequence). A phage containing genomic sequences were isolated from a library made by cloning Raji DNA partially digested with $MboI$ (ref. 13) into $\lambda 2004$ (ref. 28), initially using the mouse clone 86T1 (ref. 3) as the probe. Raji cells derive from a Burkitt's lymphoma patient²⁹. As the phage clones were derived from a B-cell library, we cannot be certain at present that the 5' end of clone $\lambda\beta 3$ is equivalent to germ-line DNA. **b**, The nucleotide sequence used to orient the C_β genes was obtained by subcloning into M13mp18 and partially sequencing the 1-kb $HindIII$ - $EcoRI$ fragment. The sequenced part is indicated in Fig. 1.

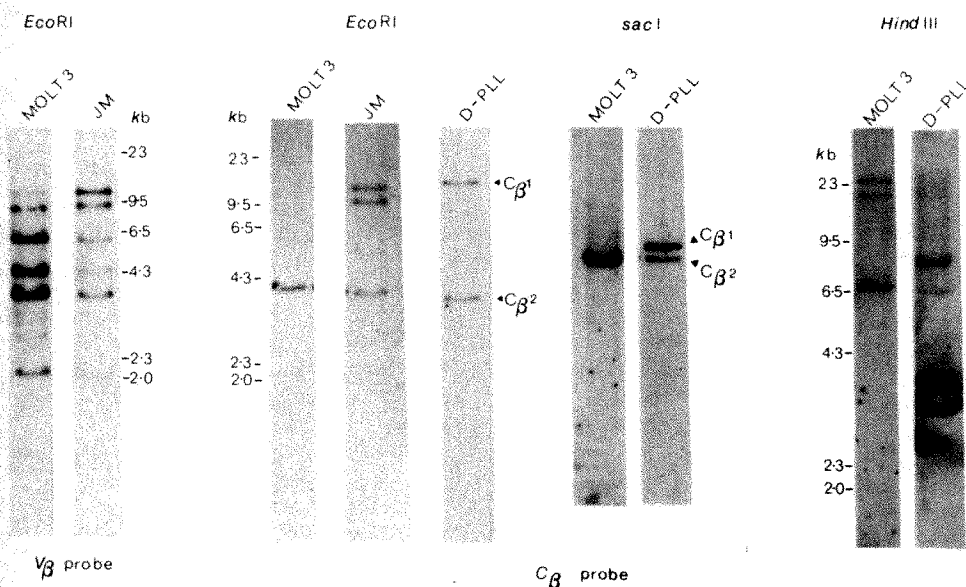
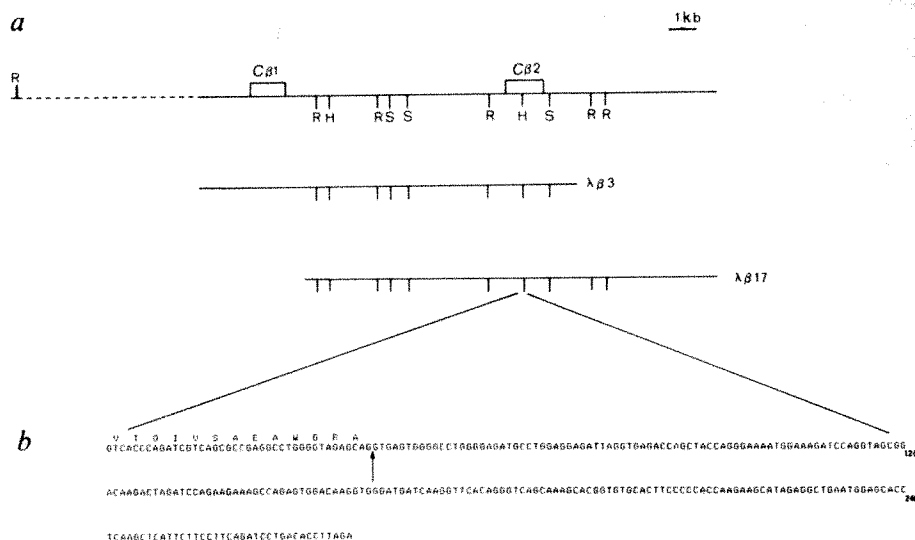


Fig. 4 Southern filter hybridizations of genomic DNA with V_β and C_β probes. Genomic DNA ($10 \mu\text{g}$) was digested with restriction enzymes, followed by electrophoresis in 0.8% agarose gels and transfer to nitrocellulose filters³⁰. The filters were subsequently hybridized to nick-translated³¹ DNA probes (specific activity $1-4 \times 10^8$ c.p.m. μg^{-1}) in $6 \times \text{SSC}$, 0.1% SDS, 5% dextran sulphate, $50 \mu\text{g ml}^{-1}$ sonicated, denatured salmon sperm DNA plus 0.2% Ficoll, 0.2% bovine serum albumin and 0.2% polyvinylpyrrolidone³². After hybridization for approximately 18 h at 65°C , the filters were washed in $0.1 \times \text{SSC}$, 0.1% SDS (5×10 min at 65°C) and autoradiographed at -70°C with pre-fogged film³³. Sizes were determined by co-electrophoresis of λ phage DNA cut with $HindIII$. D-PLL is leukaemic DNA from a patient with prolymphocytic leukaemia and possesses the germ-line C_β gene profile (unpublished data).

multiple V_β segments. Each C_β gene is functional, as $C_\beta 1$ is expressed in the JM cell line, whereas $C_\beta 2$ is rearranged and presumably expressed in MOLT-3.

There are several similarities between the T-cell receptor β -gene locus and the immunoglobulin gene families: for example, the occurrence of deletions associated with gene rearrangements and the existence of multiple inherited V segments (as well as D and J segments) giving considerable potential for diversity. The inherited diversity of human V_β genes is reasonably high as judged by filter hybridization and analysis of V-gene expression in a limited panel of T-cell lines. Perhaps the existence of separate J_β clusters associated with each C_β gene^{7,8} is another strategy required to increase diversity. The similarity of the human and murine β -chain gene organization suggests that the occurrence of two tandem and highly homologous C_β genes may have functional significance. It will be important to establish the extent of germ-line V_β and D_β diversity, to investigate V-gene expression in antigen-specific T-cell clones, and particularly to study the contribution of the α -chain genes.

We thank A. Forster and M. A. Stinson for technical help, Dr M. Davis for providing clone 86T1, Drs P. Beverley, A. Goodall and J. Minowada for cell lines, and Drs M. Owen and H. Stockinger for DNA. JM, HPB-ALL, HPB-MLT, CCRF-CEM and MOLT-3 were obtained from Dr J. Minowada, J6 from Dr P. Beverley and a second sample of MOLT-3 from Dr A. Goodall. Two different JM DNA samples were obtained from Dr H. Stockinger (one originally from Dr M. Schreier and the other from the Max-Planck-Institut, Freiburg). We also thank Dr J. G. Sutcliffe for helpful suggestions. A.T. is the recipient of an MRC Training Fellowship.

Note added in proof: We have now received a sample of MOLT-3 DNA from Dr T. Mak. The pattern of C_β gene rearrangement in this DNA is identical to that of our various JM DNA samples (by $EcoRI$ digestion). We also find that our JM DNA has a rearrangement of one immunoglobulin J_H allele (T.H.R., manuscript in preparation); the MOLT-3 DNA from Dr T. Mak has an identical J_H rearrangement whereas our own MOLT-3 DNA samples have a germ-line J_H pattern.

Received 31 August; accepted 5 October 1984.

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Monoclonal antibodies identify a cell-surface antigen associated with an activated cellular oncogene

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A variety of antigens have been identified on the surface of the malignant cell. However, identical antigens are often found on non-malignant cells of the same or different histological origin, or of a different stage of embryonic development¹⁻⁵. Many of these tumour-associated antigens appear to be only incidentally expressed on neoplastic cells. Clearly, it would be of great interest to identify cell-surface antigens whose expression is associated specifically with the transformed state and linked directly with the mechanisms responsible for transformation. The detection of activated cellular oncogenes in human and animal cancer cells by the technique of DNA transfection has allowed the isolation of genetic elements which are thought to have a critical role in malignancy^{6,7}. Here, in an effort to identify cell-surface antigens associated with the neoplastic process, we have generated hybridomas which secrete monoclonal antibodies that react specifically with cell-surface determinants found on NIH 3T3 cells transformed by transfection with a group of rat neuroblastoma oncogenes. These antibodies bind to and immunoprecipitate a phosphoprotein of relative molecular mass 185,000 (185 K) from a DNA donor rat neuroblastoma and 13 independent rat neuroblastoma DNA transfectants. There was no antibody reactivity with normal NIH 3T3 cells or with NIH 3T3 cells transformed by various other agents.

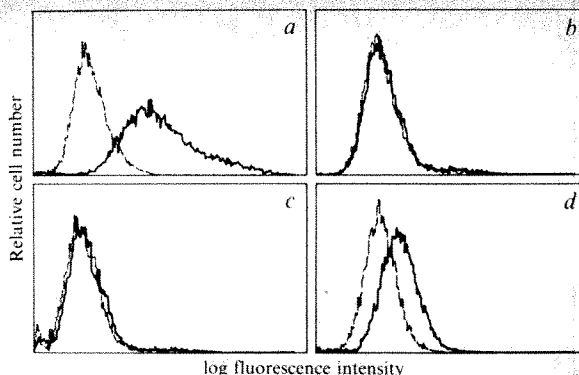


Fig. 1 FACS histograms of cells stained with monoclonal antibody 7.16.4. Histograms representing cell populations stained with monoclonal antibody and FITC-conjugated rabbit anti-mouse immunoglobulin are represented by solid lines (—) while control populations stained with FITC-conjugated rabbit anti-mouse immunoglobulin alone are represented by broken lines (---). *a*, *neu* gene transfectant cell line, B104-1-1. *b*, Normal NIH 3T3 cells. *c*, *Ha-ras* transfectant cell line, XHT-1-1a. *d*, Activated *neu* gene donor rat neuroblastoma, B104.

Methods: Tumour cell lines were grown in tissue culture dishes in Dulbecco's modified Eagle's supplemented with 10% fetal calf serum (FCS), 1% penicillin-streptomycin-Fungizone and 1% gentamycin. Cells were removed from dishes with buffered EDTA (Versene; Gibco) and washed twice in FACS medium (Hank's balanced salt solution (HBSS; Gibco) supplemented with 2% FCS, 0.1% sodium azide and 10 mM HEPES); 1×10^6 cells in 0.1 ml FACS medium were incubated with 0.1 ml of hybridoma culture supernatant for 1 h at 4°C. Cells were washed twice with FACS medium, and incubated with 0.1 ml FITC-conjugated rabbit anti-mouse immunoglobulin (Miles) diluted 1:50 in FACS medium or 1 h at 4°C. Cells were then washed twice in FACS medium and fixed in 2% paraformaldehyde-phosphate-buffered saline (PBS). Samples were run on a Coulter Epics V fluorescence-activated cell sorter. We routinely analysed 10,000 cells per sample. To control for nonspecific binding of the FITC-conjugated rabbit anti-mouse antibody to tumour cells, parallel samples were run which were stained with this reagent alone.

We have described previously a group of oncogenes found in the genomes of several ethylnitrosourea-induced rat neuroblastomas; these oncogenes induced neoplastic transformation of mouse NIH 3T3 cells when introduced by the technique of DNA transfection⁸. Inoculation of young mice with NIH 3T3 transfectants transformed by rat neuroblastoma DNA resulted in the appearance of fibrosarcomas. Sera from the tumour-bearing mice immunoprecipitated a 185K phosphoprotein (p185) that was present in all mouse cells that had acquired the rat oncogene⁹. p185 was absent from mouse cells that had been transformed by other oncogenes, including the *ras* oncogenes. Moreover, mouse cells transformed by the rat neuroblastoma oncogene lack increased levels or altered versions of the known *ras* oncogenes and their p21 products¹⁰. These data suggest that synthesis of p185 is specifically induced by the rat neuroblastoma oncogene, and that this oncogene is distinct in its behaviour from other known oncogenes. We refer to members of this family of oncogenes by the term '*neu*'.

Preliminary experiments suggested that *neu*-transfected NIH 3T3 cells displayed transformation-specific antigens on their surface. These experiments used polyclonal sera raised in mice injected with *neu*-transfected NIH 3T3 cells emulsified in Freund's adjuvant. Immunofluorescence tests showed reactivity of antiserum with intact transfected cells which was not affected by extensive pre-absorption on normal NIH 3T3 cells (data not shown). Based on these results, we used indirect immunofluorescence as a screening technique in our efforts to develop monoclonal antibodies reactive with cell-surface determinants on *neu*-transfected cells. Briefly, C₃H/HeJ mice were repeatedly immunized with NIH 3T3 transfectants transformed by the *neu* oncogene (cell line B104-1-1), emulsified in Freund's adjuvant.

Table 1 Expression of p185 on murine tumour cell lines

Cell line	Description	Immuno-fluorescence
NIH 3T3	Contact-inhibited mouse fibroblast	-
B104-2	1 ⁰ Transfectant of B104 rat neuroblastoma DNA	+
B104-1-1	2 ⁰ Transfectant of B104 DNA	++++
B104-1-2	2 ⁰ Transfectant of B104 DNA	+
B103-1	1 ⁰ Transfectant of B103 rat neuroblastoma DNA	+++
B103-5	1 ⁰ Transfectant of B103 DNA	++
B103-1-1	2 ⁰ Transfectant of B103 DNA	++
B103-1-2	2 ⁰ Transfectant of B103 DNA	+
B50-1	1 ⁰ Transfectant of B50 rat neuroblastoma DNA	+
B50-6a-2	2 ⁰ Transfectant of B50 DNA	++++
B50-6a-3	2 ⁰ Transfectant of B50 DNA	++
B82-1	1 ⁰ Transfectant of B82 rat neuroblastoma DNA	+
B82-3	1 ⁰ Transfectant of B82 DNA	+++
B82-3-2	2 ⁰ Transfectant of B82 DNA	+
XHT-1-1a	2 ⁰ Transfectant of v-Ha-ras proviral DNA	-
RSV 3T3	RSV-transformed NIH 3T3	-
MSV 3T3	Moloney sarcoma virus-transformed NIH 3T3	-
HaSV 3T3	Harvey sarcoma virus-transformed NIH 3T3	-
PyD2	Polyoma virus-transformed mouse fibroblast	-
PyBR	Polyoma virus-transformed mouse fibroblast	-
SVD2	SV40-transformed mouse fibroblast	-
S1509a	3-Methylcholanthrene-transformed mouse fibroblast	-
YAC	Mo MuLV-induced lymphoma	-
SP2	Mineral oil-induced plasmacytoma	-

Cell lines were prepared for FACS analysis on a Coulter Epics V Cell Sorter using saturating amounts of monoclonal antibody 7.16.4 as described in Fig. 1 legend. Immunofluorescence was quantitated by subtracting the median fluorescence channel of populations stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin alone (negative control) from the median fluorescence channel of populations stained with monoclonal antibody 7.16.4 followed by FITC-conjugated rabbit anti-mouse (positive staining). The resulting net fluorescence was expressed according to the following scale: -, less than two channels; +, 5-10 channels; ++, 11-15 channels; +++, 16-20 channels; +++++, >20 channels. 1⁰ and 2⁰ indicate primary or secondary transfectants created by passing the oncogene through one or two sequential cycles of DNA transfection in NIH 3T3 mouse fibroblasts. MoMuLV, Moloney murine leukaemia virus.

Spleens from immune mice were fused with the aminopterin-sensitive NS-1 myeloma line, and hybridomas were selected in hypoxanthine-aminopterin-thymidine media. Culture supernatants from growing hybridomas were initially screened for the presence of antibody capable of binding B104-1-1 cells by indirect immunofluorescence using fluorescence activated cell sorting (FACS). Positive supernatants were then tested for specificity by determining whether they contained antibody capable of binding normal NIH 3T3 cells, or NIH 3T3 cells transformed by transfection with Harvey sarcoma virus proviral DNA (cell line XHT-1-1a).

To date, we have screened ~3,000 hybridomas and have identified five that produce monoclonal antibodies capable of specifically binding B104-1-1 cells. One of these hybridomas, that produces an antibody termed 7.16.4, will be described in detail. It secretes immunoglobulin of the IgG2a isotype and has maintained production of specific antibody through three cycles of limiting dilution cloning and two cycles of soft agar cloning. Figure 1 shows FACS histograms representing the binding of monoclonal antibody 7.16.4 to several different cell lines. FACS

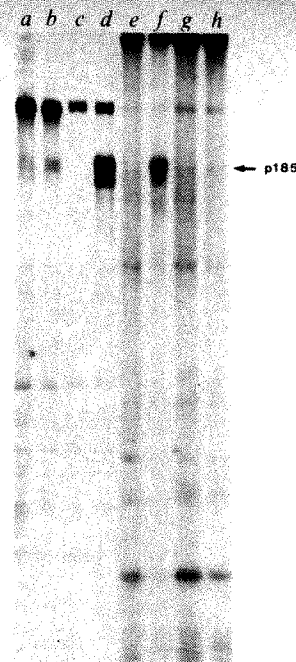


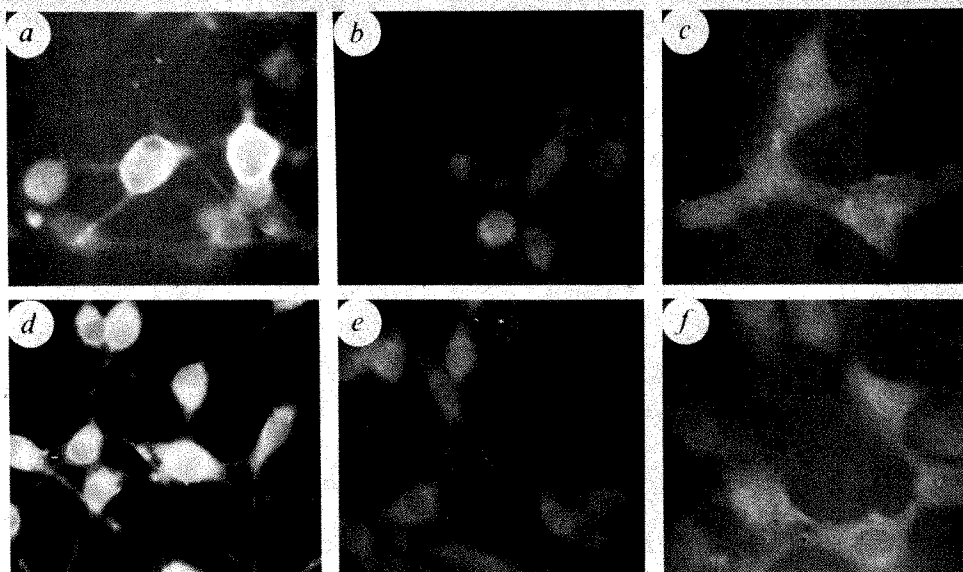
Fig. 2 Immunoprecipitation of metabolically labelled p185 by monoclonal antibody 7.16.4. *a-d*, ³⁵S-cysteine-labelled cell lysates: *a*, NIH 3T3 lysate precipitated with normal mouse serum (NMS); *b*, NIH 3T3 lysate precipitated with monoclonal antibody 7.16.4; *c*, *neu* gene transfectant B104-1-1 lysate precipitated with NMS; *d*, B104-1-1 lysate precipitated with 7.16.4. *e-h*, ³²P-labelled cell lysates: *e*, B104-1-1 lysate precipitated with NMS; *f*, B104-1-1 lysate precipitated with 7.16.4; *g*, NIH 3T3 lysate precipitated with NMS; *h*, NIH 3T3 lysate precipitated with 7.16.4.

Methods: For labelling with ³⁵S-cysteine, 10⁶ cells were seeded in 100-mm culture dishes and labelled for 18 h in 2 ml minimal essential medium (MEM) containing 0.1 the usual amount of cysteine, 2% dialysed FCS and 500 μCi ³⁵S-cysteine (77 Ci mmol⁻¹; NEN). For labelling with ³²P, 3 × 10⁵ cells were seeded in 60-mm tissue culture dishes and incubated for 18 h in 0.8 ml phosphate-free Dulbecco-Vogt modified Eagle's medium containing 4% FCS and 0.4 mCi ³²P (carrier-free; NEN). Cells were lysed in phosphate-buffered RIPA buffer containing 1 mM ATP, 2 mM EDTA and 20 mM sodium fluoride, and immunoprecipitates were prepared and washed as described elsewhere²². One-third of each lysate was incubated with 1 μl of normal mouse serum or 60 × concentrated 7.16.4 culture supernatant at 4 °C for 60 min. Sheep anti-mouse immunoglobulin (1 μl; Cappel) was added to each sample, and incubation continued for 30 min. Immune complexes were pelleted using fixed Protein A-bearing *Staphylococcus aureus* and washed. Samples were analysed by SDS-polyacrylamide gel electrophoresis in 7.5% acrylamide-0.17% bisacrylamide gels²². The gels were treated for fluorography and exposed to preflashed Kodak X-Omat AR film for 10 days.

can quantitate antibody binding to particular cell populations by immunofluorescence, displaying more brightly stained populations (those which bind more antibody) to the right of less brightly stained populations. Figure 1 shows strong binding of the antibody to cell line B104-1-1 (*a*), and significant, although weaker, binding to the DNA donor, the rat neuroblastoma cell line, B104 (*d*). Antibody 7.16.4 binds neither to nontransformed NIH 3T3 cells (Fig. 1*b*) nor to NIH 3T3 cells transformed by a Harvey *ras* (Ha-*ras*) oncogene (Fig. 1*c*). Thus, this antibody is directed against neither a normal NIH 3T3 cell-surface antigen nor an antigen associated in a nonspecific fashion with either proliferation or neoplastic transformation of murine cells. Antibody 7.16.4 appears to recognize a cell-surface antigenic determinant specifically expressed by cells containing the *neu* oncogene.

Immunofluorescent staining for FACS analysis was performed on cells that were initially >90% viable as determined by Trypan blue exclusion. However, we considered it formally possible that cell death during antibody staining might allow antibody to enter dead cells and cause cell fluorescence due to

Fig. 3 Indirect immunofluorescent staining of B104-1-1 and NIH 3T3 cells. *a-c*, Paraformaldehyde-fixed cells: *a*, *neu* gene transfectant B104-1-1 cells stained with 7.16.4 plus FITC-conjugated rabbit anti-mouse antibody; *b*, B104-1-1 cells stained with FITC-conjugated rabbit anti-mouse antibody alone; *c*, NIH 3T3 cells stained with 7.16.4 plus FITC-conjugated rabbit anti-mouse antibody. *d-f*, Methanol-fixed cells: *d*, B104-1-1 cells stained with 7.16.4 plus FITC-conjugated rabbit anti-mouse antibody; *e*, B104-1-1 cells stained with FITC-conjugated rabbit anti-mouse antibody alone; *f*, NIH 3T3 cells stained with 7.16.4 plus FITC-conjugated rabbit anti-mouse antibody. NIH 3T3 cells fixed with either paraformaldehyde or methanol and stained with FITC-conjugated rabbit anti-mouse antibody alone showed staining comparable to that in *c* and *d* (data not shown).



Methods: Cells were grown on glass coverslips for 48 h, then the coverslips were washed in PBS and fixed with either 2% paraformaldehyde-PBS or absolute methanol for 10 min at room temperature. Coverslips were then washed in FACS medium and incubated with 10-fold concentrated 7.16.4 culture supernatant or FACS medium (for controls) for 30 min at 4 °C. They were then washed in FACS medium and stained with FITC-conjugated rabbit anti-mouse immunoglobulin (diluted 1:50; Miles) for an additional 30 min. The coverslips were extensively washed in FACS medium, washed in PBS, and antibody was fixed *in situ* by a 10-min incubation in 2% paraformaldehyde-PBS. Coverslips were mounted on glass slides and photographed using 20-s exposures to Kodak Tri-X pan film with a Leitz fluorescence microscope at $\times 400$ magnification.

the binding of intracellular rather than cell-surface antigenic determinants. We excluded this possibility by performing FACS analysis on live cells stained with fluorescent antibodies in the presence of propidium iodide. Propidium iodide is excluded from live cells, but enters dead cells, where it intercalates with nucleic acids and emits a red fluorescence when excited by the FACS 488-nm argon laser¹¹. By setting the cell sorter to exclude cells displaying such red fluorescence, we confirmed that monoclonal antibody 7.16.4 reacts with a determinant on the surface of intact B104-1-1 cells (data not shown).

The specificity of the antibody was further examined by immunoprecipitation of metabolically labelled cell lysates. Figure 2 demonstrates that monoclonal antibody 7.16.4 immunoprecipitates a phosphoprotein with a relative molecular mass of ~ 185 K (p185) from the *neu*⁺ NIH 3T3 transfectant B104-1-1 (Fig. 2*d, f*). Several other faint bands are also visible in both normal mouse serum immunoprecipitates and monoclonal antibody 7.16.4 immunoprecipitates of B104-1-1 cell lysates (Fig. 2*c-f*). A faint band of about the same size is seen in NIH 3T3 lysates immunoprecipitated with either normal mouse serum or monoclonal antibody (Fig. 2*a, b, g, h*); because this band is slightly different in molecular weight from p185, and is also precipitated by normal mouse serum, we conclude that there is no specific precipitation of p185 from normal NIH 3T3 cells by monoclonal antibody 7.16.4. The 185K phosphoprotein identified by antibody 7.16.4 appears to be identical to the *neu* oncogene-associated p185 identified previously using polyclonal sera (data not shown).

FACS analysis, using monoclonal antibody 7.16.4, of a large number of murine cells transformed by a variety of agents has revealed that p185 is expressed only in cells transformed by *neu* oncogenes (Table 1). By this test, NIH 3T3 cells transformed by several retroviruses containing known oncogenes, including *src*, *mos* and *ras*, lack p185. Similarly, mouse fibroblast lines transformed by the papovaviruses simian virus 40 (SV40) and polyoma virus, as well as the chemical carcinogen 3-methylcholanthrene, are also not stained by monoclonal antibody 7.16.4. Murine tumours of non-fibroblast histological origin, such as the SP₂ plasmacytoma and the YAC T-cell lymphoma, also fail to bind anti-p185 monoclonal antibody. In contrast, all of 13 NIH 3T3 transfectants transformed by *neu* oncogenes contain significant amounts of cell-surface p185, as determined by antibody binding. It is interesting that the level of p185

expression varies somewhat between transfectants; cell lines that show the highest expression of p185 as determined by immunofluorescence also contain the most p185 as determined by quantitative immunoprecipitation (data not shown). Although a very high level of p185 is seen in some transfectants, such a high level does not seem to be necessary for oncogenic transformation of NIH 3T3 cells. The absolute correlation between the expression of cell-surface p185 and the presence of rat *neu* oncogenes in murine transfectants suggests, but does not prove, that p185 is a *neu* oncogene product.

Some oncogene-encoded proteins have been localized to particular functional regions within the cell: for example, the Rous sarcoma virus (RSV) transforming gene product, pp60^{src}, is found in high concentration adjacent to cellular adhesion plaques¹². In an effort to determine whether such cytoplasmic compartmentalization exists for p185, we performed indirect immunofluorescence on non-permeabilized and permeabilized B104-1-1 cells grown on coverslips (Fig. 3). Apparently, p185 is distributed in a diffuse pattern on the extracellular face of the plasma membrane of paraformaldehyde-fixed cells (Fig. 3*a*). In contrast, cells permeabilized by fixation in absolute methanol display diffuse antibody binding throughout the cell, and are particularly well stained in the perinuclear region of the cytoplasm (Fig. 3*d*). It remains to be determined whether these results reflect compartmentalization of p185 precursor proteins undergoing processing on membrane-associated ribosomes or in the Golgi apparatus, or whether they are simply a result of the thicker cross-section in this region of the cell. p185 does not appear to be specifically associated with sites of cellular adhesion or cell-cell contact.

Several proteins have been associated with the various activated oncogenes described in non-virally-induced tumour cells and cell lines¹³⁻¹⁵. However, none of these proteins is similar in size to the 185K antigen associated with the *neu* oncogene and none has been identified as a cell-surface protein. Furthermore, none of the proteins known to be encoded by retroviral oncogenes has physical properties identical to those of p185, although the gene products of *v-fms* and *v-erb-B* and their cellular homologues may also have portions of the respective molecules exposed on the cell surface¹⁶⁻¹⁸. The data presented here and in ref. 10 strongly suggest that p185 is a *neu* oncogene product.

The cell-surface localization of p185 may offer insight into a

potential mechanism of oncogene action. p185 has many characteristics similar to those of the receptors for epidermal growth factor (EGF)¹⁹ and platelet-derived growth factor (PDGF)²⁰, including approximate molecular weight, plasma membrane localization and phosphorylation. p185 is also a glycoprotein (D.F.S., unpublished results), as are the EGF and PDGF receptors. Waterfield and colleagues have presented persuasive evidence that the viral *erb-B* oncogene represents a truncated EGF receptor gene which lacks the extracellular EGF-binding domain but nonetheless delivers to the cell a mitogenic signal which results in neoplastic transformation²¹. The *neu* gene product p185 may also be an altered growth factor receptor which transforms cells by a mechanism similar to that of *v-erb-B*. Its localization to the cell surface and association with oncogene

activation suggest that p185 may prove useful as a potential target for directing monoclonal antibodies against tumours containing activated *neu* oncogenes; we are presently investigating this possibility.

We thank Mr Jim Throp for preparation of the manuscript, Willis Chung for technical assistance in the early phases of this study, and Drs Ann Carroll, Adam Lowy and John Monroe for thoughtful criticism. J.A.D. was supported by NIH grant 5-T32-GM07753 from the Medical Scientist Training Program at Harvard Medical School. D.F.S. was supported by postdoctoral fellowships from the Damon Runyon Cancer Fund and the NIH. This work was supported by grant CA 14723 from the NCI and by an American Cancer Society Faculty Award to M.I.G.

Received 18 June; accepted 19 September 1984.

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Therapy with monoclonal antibodies by elimination of T-cell subsets *in vivo*

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A major aim in immunology has been to understand how the immune system evokes characteristic responses to infection, foreign tissue grafts and tumours. The current view of immunoregulation is based mainly on studies of lymphocyte subsets, either *in vitro*¹⁻³ or by adoptive transfer to irradiated recipients⁴. Many reagents are available for defining T-cell subsets⁵⁻⁸, but only recently have there been helper T-cell-specific antibodies^{9,10} against the mouse equivalent of the Leu3/T4 (man) and W3/25 (rat) antigens. It is clear that monoclonal antibodies¹¹ will eventually replace anti-lymphocyte globulin for immunosuppression in organ grafting^{12,13}, but although there has been some clinical success¹⁴⁻¹⁶, most monoclonal reagents cause only transient reductions in their target cells *in vivo*¹⁷⁻²⁰. This uncertainty in the potency of monoclonal antibodies has led some workers to consider them as targeting agents for such highly cytotoxic drugs as ricin A (ref. 21). We show here that unmodified monoclonal antibodies can be extremely effective at depleting cells *in vivo* and can be used for the selective manipulation of different aspects of the immune response.

The observation that two different rat IgG2b anti-mouse Thy-1 antibodies were potently immunosuppressive when administered *in vivo*, compared with a range of other monoclonal antibodies of the same specificity but different isotypes^{22,23}, suggested that the antibody isotype might be an important factor for serotherapy. We therefore prepared²⁴ rat monoclonal antibodies to several mouse T-cell antigens using the Y3/Ag1.2.3 myeloma²⁵ as fusion partner and compared these for their ability to suppress the *in vivo* antibody response to sheep red blood cells (Fig. 1). Adult thymectomized mice were used so that new T cells would not be produced after monoclonal antibody depletion, and immunosuppression could then be measured in the absence of any short-term effects of the antibody. Only the IgG2b antibodies recognizing an antigen expressed on the helper T cells (Thy-1, Lyt-1 and L3/T4) were effective at eliminating the antibody response. Six rat anti-Thy-1 monoclonal antibodies of other isotypes (IgM, IgG2a and IgG2c) and one IgG2a

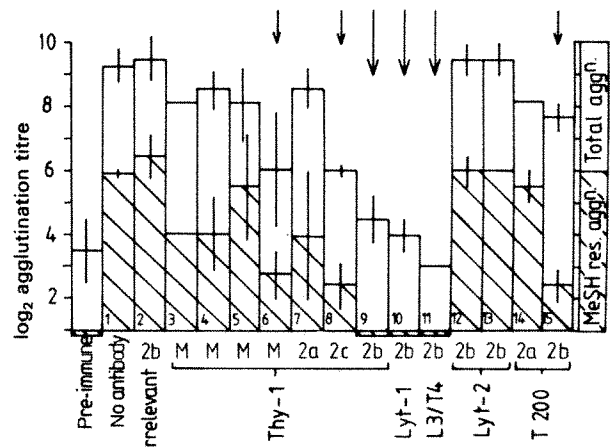


Fig. 1 Suppression of the antibody response to SRBCs. Male CBA mice were thymectomized at 5-6 weeks of age and randomly selected 3 weeks later for monoclonal antibody administration, given as two intravenous injections, 1 week apart, of 0.2 ml of monoclonal ascitic fluid [from (LOU × DA)_{F1} rats], equivalent to ~0.5 mg of active antibody in the following groups: 1, no antibody ($n=10$); 2, irrelevant IgG2b ($n=2$), YTH 89.1 anti-human glycoprotein A; G. Hale, personal communication); 3-6, four different IgM anti-mouse Thy-1²³ (YTS 27.3, $n=2$; YTS 109.8, $n=2$; YTS 198.3, $n=2$; YTS 148.3, $n=4$); 7-9, IgG2a (YTS 111.4, $n=2$), IgG2c (YBM 29.2, $n=4$), IgG2b (YTS 154.7, $n=4$) anti-mouse Thy-1²³; 10, IgG2b anti-mouse Lyt-1 ($n=4$; YTS 121.5 which cross-inhibits 53-7.313 (ref. 7 and our unpublished observation); 11, IgG2b anti-mouse L3/T4 ($n=4$; YTS 191.1 which is present on only the Lyt-2 negative peripheral T cells (Table 1), 85-90% of thymocytes and <2% of bone marrow cells, reacts with a protein of relative molecular mass 55,000 as determined by 'Western blots' (ref. 45 and our unpublished observations) and is therefore similar to GK1.5 (ref. 10); 12, 13, two IgG2b anti-Lyt-2 [YTS 169.4, $n=6$; YTS 172.3, $n=6$, which cross-inhibit 53-6.72 (ref. 7 and our unpublished observation)]; 14, 15, IgG2a (YBM 42.2 (ref. 46), $n=2$) and IgG2b (YW 62.3, $n=2$, which cross-inhibits YBM 42.2, our unpublished observation) anti-T200 ('leukocyte common antigen'⁴⁷). Animals were given 0.2 ml of 10% SRBCs intraperitoneally 3 weeks after monoclonal antibody (except for preimmune group, $n=6$). These were bled from the tail vein after 10 days and the serum agglutination (aggⁿ) titre determined with SRBCs alone (total IgM and IgG response) or with SRBCs in the presence of 0.1 M 2-mercaptoethanol (MeSH-resistant IgG response). Substantial immunosuppression is indicated by the arrows.

anti-T200 were far less effective than the IgG2b reagents of the same specificities.

The observed immunosuppression could be accounted for by depletion of helper or total T cells (Table 1). At various times from 1 week to 3 months after the original injection of monoclonal antibody, groups of treated, or control thymectomized, mice were compared for the expression of various T-cell markers using the cytofluorograph. All of the rat IgG2b antibodies gave substantial and long-term depletion (5 weeks post-antibody in Table 1) of their target T cells. Residual rat immunoglobulin could not be detected and an anti-globulin response was observed by day 10 (in all but YTS 191.1-treated mice, results not shown). This means that suppression was unlikely to be due to blocking of function, temporary changes in the pattern of lymphocyte circulation or 'antigenic competition'²⁶, which could contribute to the short-term effects described in other systems^{23,27}, such as that of LeGros *et al.*²⁸.

Selective manipulation of the major T-cell subsets could be readily achieved with the IgG2b monoclonal anti-L3/T4 or -Lyt-2 antibodies (Table 1). For example, if we consider the YTS 169.4 (anti-Lyt-2) treated group, the Lyt-2-positive cells were reduced to 0.2%, which represents a specific depletion of at least 96% compared with thymectomized controls. Elimination of cells was also effective in the lymph-nodes, with 14.5% Lyt-2-positive T cells in controls compared with 1.2% in treated mice (results not shown). The loss of Thy-1, L3/T4 and Lyt-1 in these treated mice was consistent with a reduction in only the Lyt-2-positive T cells. Over a 12-month observation period, there was no observable change in Lyt-2 expression. This demonstrates that any remaining Lyt-2-positive T cells were unable to repopulate the animal and that, in these conditions, the mature Lyt-2-negative phenotype was stable and irreversible, in contrast to some suggestions of phenotypic switching²⁹. Similar long-term depletions were observed in the YTS 191.1 (anti-L3/T4) treated mice, although we have not followed these animals beyond 6 months post-injection. It is possible that further injections of antibodies are required to maintain depletion during a very strong antigenic challenge, where small numbers of residual T cells might be induced to proliferate.

Immunological responses in T-cell subset-depleted mice were those expected considering the surface phenotype of the remaining T cells. Mice depleted of L3/T4-positive cells did not make an antibody response to sheep cells (Fig. 1) or the injected rat immunoglobulin (results not shown), and were also unable to develop delayed hypersensitivity (HSV-DH, Table 1) to herpes simplex virus (recently implicated as an important effector mechanism in immunity to such viruses³⁰ and in allograft rejection³¹). Mice depleted of either subset (L3/T4 or Lyt-2) were unable to produce cytotoxic T cells to allogeneic targets *in vitro* (Table 1, Fig. 2). This is because the generation of cytotoxic T cells depends on the presence of both helper (Lyt-2⁻) and cytotoxic precursor (Lyt-2⁺) cells³²; simple mixing of the cells from the L3/T4- and Lyt-2-depleted animals at the start of the culture period reconstituted the response (Fig. 2). Therefore, animals can be independently depleted of the 'helper' or 'cytotoxic' T-cell lineages by administering monoclonal antibodies to L3/T4 and Lyt-2. These animals have remained functionally depleted for at least 6 months after monoclonal antibody injection. Previously, this could only be achieved, albeit inefficiently, by using populations of T cells depleted of Lyt-1⁺ or Lyt-2⁺ subsets *in vitro* to reconstitute lethally irradiated, thymectomized mice⁴. Such animals may have a contribution from radio-resistant T cells³³, and complications associated with irradiation are avoided by the *in vivo* antibody treatment. The major advantage of using subset-specific monoclonal antibodies directly in animal models is that it should be possible to apply the rules learned in, for example controlling allograft rejection, to therapy in man.

It is still not entirely clear which T cells are responsible for the rejection of skin grafts in mice^{31,34,35}, partly perhaps because of some confusion over Lyt-1 expression³³. We used CBA mice depleted of either L3/T4- or Lyt-2-positive T cells to determine the cells required to reject fully mismatched (BALB/c) or H-2-matched (minor antigens mismatched, B10.BR) skin grafts (Fig. 3). In both cases, Lyt-2 depletion did not delay rejection, while L3/T4 depletion led to greatly increased survival of the grafts. These results were confirmed using normal CBA mice (with a thymus) given a 14-day course of antibody to cover the initial

Table 1 Depletion of T-cell subsets *in vivo* by serotherapy with monoclonal antibodies

Antibody treatment	% Positive spleen cells					% Specific depletion	HSV-DH ($\times 10^{-2}$ mm)	Allo-CTL (% control)
	Thy-1 (154.7)	Lyt-1 (121.5)	Lyt-2 (169.4)	L3/T4 (191.1)	L3/T4: Lyt-2 ratio			
YTS 154.7	<u>1.6</u> (-)	2.3(+)	0.6(-)	ND	ND	90	10.4 $\pm$ 1.5	ND
YTS 121.5	2.5	<u>2.7</u> (-)	0.7(-)	ND	ND	82	5.5 $\pm$ 1.0	21
YTS 169.4	10.3	11.0	<u>0.2</u> (-)	11.3	57	96	13.0 $\pm$ 1.8	2
YTS 191.1	8.8	4.7	5.0	<u>1.5</u>	0.3	86	4.5 $\pm$ 2.0	0
None (ATX)	15.7	14.6	5.1	10.8	2.1	0	15.4 $\pm$ 2.3	100
Normal	33.5	35.0	11.4*	24.3*	2.1	ND	ND	ND

Male CBA mice were thymectomized at 5-6 weeks of age, randomly selected for injection of monoclonal antibodies and tested 3 weeks later for their responses to sheep red blood cells (SRBCs) as described in Fig. 1 legend, HSV-specific delayed hypersensitivity (DH) as described below or generation of allo-CTL as described in Fig. 2. Two mice from each of the SRBC-primed group were selected at random, 5 weeks after monoclonal antibody injection, and the spleen (and mesenteric lymph nodes, data not shown) was taken for analysis of T-cell markers. Red cells were lysed by NH_4Cl treatment and the remaining lymphocytes incubated for 1 h at 4 °C in antibody supernatants at 100 $\mu\text{g ml}^{-1}$ of medium containing 5% heat-inactivated normal rabbit serum to block Fc binding, and 0.1% NaN_3 . After washing, bound monoclonal antibody was detected by incubation with fluorescein isothiocyanate (FITC)-labelled mouse monoclonal antibodies to rat immunoglobulin light chains (MAR 18.5)⁴² and rat IgG2b (NORIG 7.16)³⁶. Cells were washed in medium containing 50 $\mu\text{g ml}^{-1}$ propidium iodide to stain dead cells, and resuspended in phenol red free medium containing 0.5% bovine serum albumin and 0.1% NaN_3 . Samples were analysed using the H50 Cytofluorograf with 2150 computer (Ortho Diagnostic) at 1,000 cells s^{-1} . Computer gating was used on forward and 90° light scatters, and on red fluorescence (propidium iodide) such that histograms of green (fluorescein) fluorescence were obtained for live, monomeric cells. Numbers show % positive cells (above fluorescence channel 40 on a linear scale of 1,000 channels: controls with the FITC-labelled anti-globulins alone gave background of 0.1-0.3% positive) and are means of the results obtained from two mice in each group analysed individually. % Specific depletion is the % depletion of cells with the phenotype of injected antibody specificity as compared with ATX controls. Also shown for comparison are results for two normal (not thymectomized) CBA mice. HSV-DH: For the induction of virus-specific delayed hypersensitivity, 10^5 plaque-forming units (PFU) HSV-1 TK⁻ (strain CL101 deficient in thymidine kinase) was injected into the ear pinna, as previously described⁴³. Seven days later the mice were challenged with 5×10^5 PFU HSV-1 TK⁻ in the contralateral pinna and the DH response was measured 24 h later as the increase in ear thickness⁴⁴. The results are expressed as the arithmetic mean $\pm$ s.d. of 5 mice per group. CBA mice receiving only the challenge inoculum, as a control for nonspecific inflammation, gave $3.8 \pm 1.2 \times 10^{-2}$ mm. Allo-CTL: % control cytotoxic T lymphocytes calculated from the effector:target ratio required to give 30% specific lysis (Fig. 2). Plus or minus in parentheses indicates respectively a $\sim 10\%$ increase $\sim$ or a 25-50% decrease in mean fluorescence of positive cells. ND, not determined. Underlining emphasizes the depleted population.

* Lyt-2+ L3/T4 gave 38.7% positive.

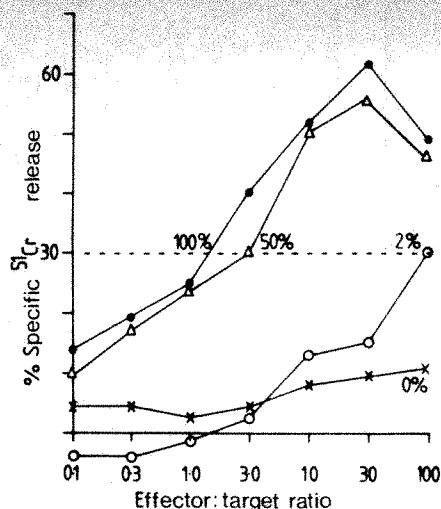


Fig. 2 L3/T4- and Lyt-2-negative mice are independently depleted of the helper and cytotoxic T-cell lineages. ●, Normal thymectomized (ATX) mice; ×, YTS 191.1 (anti-L3/T4) treated ATX mice; ○, YTS 169.4 (anti-Lyt-2) treated ATX mice; △, 50:50 mix of cells at start of culture from YTS 191.1 and YTS 169.4 ATX mice. **Methods:** Spleens were taken from two normal, two YTS 191.1 (anti-L3/T4), and two YTS 169.4 (anti-Lyt-2) treated, adult thymectomized male CBA mice, 3 weeks after monoclonal antibody administration. Responder cells from individual mice were incubated at both 2×10^6 and 5×10^6 cells ml^{-1} with 10^6 irradiated (2,500 rad) BALB/c stimulator spleen cells in 1-ml Linbro cultures, in Iscove's modified Dulbecco's medium containing 5% fetal calf serum (FCS) at 37°C for 6 days with constant rocking. Effector cells were collected and pooled from the mixed lymphocyte cultures for each animal and titrated (in triplicate) in 100- μl microtitre wells with 10^4 BALB/c spleen cells that had been previously stimulated with concanavalin A for 2 days before labelling with ^{51}Cr -labelled sodium chromate. The supernatants were removed for γ counting after 4 h at 37°C . Cytotoxic activity was measured as the % specific ^{51}Cr release, calculated as:

% Specific release

$$= \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

where the spontaneous release was from labelled targets alone, and the total release was from detergent-treated targets. Effector:target ratios were calculated on the basis of the number of cells added at the start of the mixed lymphocyte culture. Each point is the mean of two experiments. Approximate % of control activity as shown was calculated from the effector to target ratio required to give 30% specific release.

period of grafting with fully allogeneic (BALB/c) skin (mean graft survival in days: no antibody, 11 ± 2 ; anti-Lyt-2, 12 ± 2 ; anti-L3/T4, 25 ± 3 ; anti-Thy-1, 25 ± 3). Immunoperoxidase staining³⁶ of spleen sections taken on the day after rejection confirmed that the target cells were still relatively depleted. These results suggest that T cells with the phenotype Thy-1⁺, L3/T4⁺, Lyt-2⁻ are the major promoters of primary graft rejection in mice. However, Lyt-2-positive cells may have a secondary role, particularly in fully mismatched grafts, where removal of all T cells was more effective than removing just the L3/T4 cells (Fig. 3) and anti-Lyt-2 injection from day 7 of grafting in previously untreated mice did lead to delayed rejection (mean survival 18 ± 3 days compared with 13 ± 2 days in untreated controls, not shown). This confirms and extends the results obtained by adoptive transfer into lethally irradiated, thymectomized rats where the W3/25⁺ helper T cells were sufficient to cause graft rejection³⁷.

The above results demonstrate that selected rat IgG2b monoclonal antibodies can be very effective *in vivo*. These would also seem to be the best candidates for human therapy³⁸, because rat IgG2b is the only subclass known both to fix human complement^{39,40} and to activate antibody-dependent cell-mediated

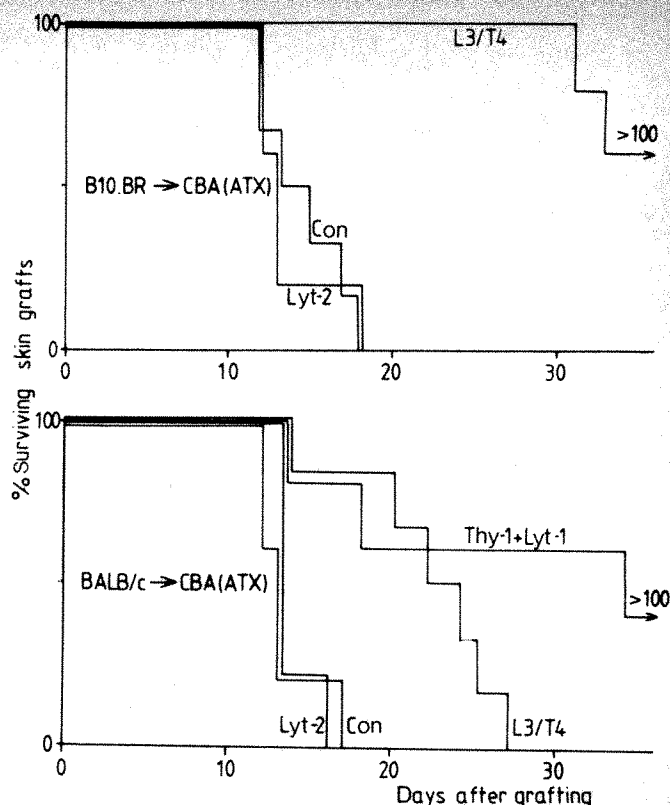


Fig. 3 Suppression of skin allograft rejection with monoclonal antibodies. Per cent surviving grafts are shown for the following groups: Con, controls with no antibody treatment; Lyt-2, YTS 169.4 (anti-Lyt-2) treated group; L3/T4, YTS 191.1 (anti-L3/T4) treated group; Thy-1 + Lyt-1, YTS 154.7 + YTS 121.5-treated group. Groups of 4–10 thymectomized CBA mice were given 2×0.2 ml of rat IgG2b monoclonal antibodies intravenously as described in Fig. 1 legend. BALB/c differing in H-2 and minor histocompatibility antigens or B10.BR (minor histocompatibility differences) tail skin was grafted to the lateral thoracic wall 4 weeks and 8 weeks after antibody treatment respectively, and covered with Vaseline-impregnated gauze and plaster bandage. The dressings were removed on day 10 and the grafts inspected every day for rejection.

cytotoxicity *in vitro*⁴¹, the latter even at very low antibody concentrations. Although most of the above experiments used large (~ 0.5 mg) amounts of antibody, YTS 169.4 is able to deplete Lyt-2 cells, and YTS 191.1 can suppress antibody responses using a total of 1–10 μg per thymectomized mouse.

Using specific monoclonal antibodies to manipulate the immune response, there are at least two ways in which fundamental aspects of immunoregulation can be investigated. First, subset-depleted mice can be used to determine the T cells involved in different aspects of bacterial or viral infections. Such animals are also a source of subset-depleted cells for *in vitro* or adoptive transfer work, with the advantage that no antibody or complement need be present at the time of the experiments. Second, T-cell subsets can be manipulated at any time during a response to foreign antigen or in autoimmune diseases, in order to probe the events which regulate the immune system as a whole. Elimination of cells *in vivo* is also a requirement for the treatment of neoplastic disease, and the above reagents are being used to develop a model for the serotherapy of leukaemias. The use of monoclonal antibodies as therapeutic agents in animal models should allow us to extend strategies for serotherapy which are directly applicable to man.

We thank Gill Martin, Penny Lovat and Jenette Phelan for their technical assistance. This work was supported by MRC grants.

Note added in proof: We have recently shown that Lyt-2⁺ cells may have a major role in graft rejection when the animals have been previously primed to the graft antigens.

Received 5 June; accepted 10 September 1984.

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Table 1 The 18-point scale used for assessing neurological function in cats examined 6 h after middle cerebral artery occlusion

Neurological response	Score
Motor function	
Cat walks with normal gait—no neurological deficit	6
Cat walks with abnormal gait, has mild hemiparesis	5
Cat barely walks with moderate hemiparesis	4
Cat unable to walk with moderate hemiparesis	3
Cat unable to walk with severe hemiparesis	2
Cat unable to walk with hemiplegia	1
Sensory function	
Cat responds appropriately to tactile and noxious stimuli	5
Cat responds appropriately to noxious limb stimuli only	4
Inappropriate response to noxious limb stimulation only	3
Reflex response to noxious limb stimulation only	2
No response to noxious limb stimulation	1
Level of consciousness	
Awake and alert	5
Awake and alert with lack of spontaneous movements	4
Drowsy, responds only to noxious stimuli	3
Stuporous, minimal response to noxious stimuli	2
Comatose	1
Pupillary response	
Unilaterally reactive to light	2
Unilaterally unreactive to light	1

osmotic pump through a small midline lumbar incision, and the cat allowed to awaken. Six hours after occlusion, sensory and motor function, consciousness and pupillary reaction to light were graded on an 18-point scale (Table 1) by two persons who did not know to which group the cat had been assigned. The cats then received an intraperitoneal injection (2 ml) of one of the following solutions: (1) sterile normal saline; (2) Leu-enkephalin, 750 $\mu\text{g kg}^{-1}$ (1.35 $\mu\text{mol kg}^{-1}$) in saline; (3) dynorphin(1-13), 2 mg kg^{-1} (1.25 $\mu\text{mol kg}^{-1}$) in saline; or (4) dynorphin(3-13), 2 mg kg^{-1} (1.43 $\mu\text{mol kg}^{-1}$) in saline. Neurological assessment was repeated 20 min after injection.

Under ketamine sedation, an osmotic pump (Alzet 2MLI, Alza Corporation) was implanted in the subcutaneous pocket to deliver saline at 10 $\mu\text{l h}^{-1}$, Leu-enkephalin at 20 $\mu\text{g h}^{-1}$ (36 nmol h^{-1}), dynorphin (1-13) at 50 $\mu\text{g h}^{-1}$ (31 nmol h^{-1}), or dynorphin (3-13) at 50 $\mu\text{g h}^{-1}$ (36 nmol h^{-1}). The pumps were coded so that each cat received the drug with which it had been injected earlier.

The cats were housed in metabolic cages at constant temperature and humidity and were examined frequently. Penicillin G, 1 MU per day, was administered intramuscularly; lactated Ringer's solution was injected subcutaneously to provide adequate daily fluid maintenance until the cat began to eat and drink. So long as the cats were alive, their neurological condition was assessed daily by the battery of tests shown in Table 1; 7 days after occlusion all the cats were killed. The brain was removed and a coronal section made at the optic chiasm. The slices were incubated in a 2% solution of 2,3,5-triphenyl-tetrazolium chloride (TTC) for 25 min^{18,19}. Colour slides were made of the stained slices, and the affected hemisphere and infarcted area were traced from projected images of the slide. Using a digitizing pad (BIT PAD ONE, Summagraphics, Fairchild, Connecticut) and a planimetry program on a PDP-11/23 computer, infarct size was calculated as the percentage of infarcted tissue relative to the entire hemisphere.

Neurological scores before and 20 min after acute injection of the peptides were compared by non-parametric analysis of variance (Kruskal-Wallis) followed by *post hoc* individual comparison, and the infarct sizes were compared by one-way analysis of variance. Kaplan-Meier survival curves were constructed and Gehan's statistics used to compare the survival between the groups.

Dynorphin(1-13) improves survival in cats with focal cerebral ischaemia

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Since the discovery of opiate receptors in the central nervous system (CNS)¹⁻³, it has become apparent that endogenous opiate ligands are involved in CNS function. Most attention has focused on their role in modulating pain, but they have also been implicated in various physiological functions and in disease states. We are concerned with evidence that endogenous opioid peptides may also contribute to the neurological deficits arising from cerebral ischaemia^{4,5}. Dynorphin, which is widely distributed in the brain and pituitary^{6,7}, has been reported to produce unusual motor and behavioural effects⁸⁻¹¹ and may act as a regulatory neuropeptide, not as a classical opiate agonist or antagonist¹²⁻¹⁵. We have therefore administered to cats in which the right middle cerebral artery had been occluded both dynorphin (1-13) and analogue and control materials. We find that dynorphin (1-13) prolongs survival.

Forty-two adult male cats (3-5 kg) assigned randomly to one of four groups were anaesthetized with halothane and subjected to transorbital occlusion of the right middle cerebral artery¹⁶. A subcutaneous pocket was created for later placement of an

Four additional cats underwent detailed studies of systemic changes associated with acute subcutaneous administration of dynorphin (1-13). After middle cerebral artery occlusion, blood pressure, heart rate, respiration and core temperature were monitored. The radiolabelled microsphere technique of Heyman *et al.*²⁰ was used to determine cardiac output and cerebral blood flow before and after subcutaneous administration of dynorphin (1-13).

Table 2 Survival after middle cerebral artery occlusion in cats

	<24 h	24-48 h	1 week
Saline	0/12 (0%)	0/12 (0%)	0/12 (0%)
Dynorphin (1-13)	7/10 (70%)	6/10 (60%)	6/10 (60%)*
Dynorphin (3-13)	2/10 (20%)	0/10 (0%)	0/10 (0%)
Leu-enkephalin	2/10 (20%)	0/10 (0%)	0/10 (0%)

* $P < 0.001$ compared with control at 1 week.

All cats in the control group died within 48 h of middle cerebral artery occlusion, whereas 60% of the cats that received dynorphin (1-13) survived for 1 week with minimal neurological deficits (Table 2). The small improvements in survival with dynorphin (3-13) and Leu-enkephalin were not statistically significant. There was no statistically significant effect on neurological function or infarct size in any group. Dynorphin (1-13) did not alter blood pressure, heart rate, cardiac output or regional cerebral blood flow.

The observation that dynorphin (1-13) does not influence systemic variables or regional cerebral blood flow suggests that its effects are mediated directly in the CNS. The ineffectiveness of dynorphin (3-13), which lacks the opioid properties of dynorphin (1-13), suggests that the capacity of the latter to enhance survival is mediated through opiate receptors.

Despite reports of the effect of peripherally administered peptides in the CNS²¹, the mode of action of dynorphin (1-13) in this study is unclear. Many peptides, such as the enkephalins, thyrotropin-releasing hormone and somatostatin, penetrate the blood-brain barrier poorly²², but it has also been suggested that even peptides with low brain uptake can enter the brain in appreciable quantities if high plasma concentrations are maintained for long enough²³. Continuous infusion of dynorphin (1-13) by subcutaneously implanted osmotic pump may have achieved this goal.

Our results provide further evidence for the involvement of endogenous opioid peptides in the pathophysiology of cerebral ischaemia. The protective effect of dynorphin (1-13) against death from focal cerebral ischaemia demonstrated here warrants further investigation and suggests possible clinical use of dynorphin (1-13) and its related analogues for human stroke.

This work was supported in part by grant no. NS20620-01 from the National Institute of Neurological and Communicative Diseases and Stroke of the National Institutes of Health. The peptides used in this study were a gift from Peninsula Laboratories. The radiolabelled microsphere study was performed with the help of Dr. Abraham M. Rudolph. We thank Bruce D. Payne for technical assistance and Stephen Ordway for editorial assistance.

Received 1 March; accepted 1 September 1984.

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Male sexual differentiation in mice lacking H-Y antigen

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The sexual phenotype of an adult mammal depends on whether the fetal gonad has differentiated as a testis or as an ovary. Because individuals of XY or XXY sex chromosome constitution develop as males, while XX and XO individuals develop as females, the presence of a Y chromosome seems normally to be required for testis differentiation and its absence to be necessary for differentiation of an ovary. The nature of the hypothetical Y-dependent substance responsible for masculinization of the indifferent gonad has been a matter for debate. A male-specific transplantation antigen, H-Y, has been known for many years^{1,2}, and more recently a serologically detected antigen, also male-specific, has been reported³. Those who believe that the two are antigenically distinct^{4,5} refer to the latter as SDM (serologically detected male) antigen, but many refer to both as H-Y antigen⁶⁻⁸. The hypothesis⁹ that H-Y is itself the Y-dependent testis inducer, although supported by little or no direct evidence^{4,10}, is economical and hence attractive. H-Y antigen is frequently stated to be the substance responsible for primary sex determination (for example, see ref. 11). We report here that H-Y is absent from certain mice that develop testes and are of indisputably male phenotype, hence this transplantation antigen is unlikely to be responsible for testis determination.

The mice examined form part of a stock segregating for *Sex-reversed* (*Sxr*)^{12,13}. *Sxr* causes XX embryos to develop as phenotypically normal though sterile males; XY carrier males are now known to possess an extra copy, transposed to a position distal to the X-Y pairing region^{14,15}, of a region of the Y chromosome located near the centromere (Fig. 1). Because of its new location, the transposed region is transferred to the X chromosome by crossing-over during male meiosis¹⁶⁻¹⁸ and, as the resulting X/X^{Sxr} mice develop as males, the region is presumed to include the testis-determining factor *Tdy* (Fig. 1). However, the masculinizing activity can be partly or wholly suppressed if the *Sxr*-carrying X chromosome is partnered by the differentially active T(X; 16)16H X-autosome translocation (T16H)^{19,20}. T16H/X^{Sxr} mice may develop as females, males or hermaphrodites; we presume that they are mosaics of female-determining cells in which the X-inactivation process has spread into the translocated *Sxr* region, and male-determining cells in which *Sxr* is still expressed. The sexual phenotype of an individual would then depend on the proportions of the two types of cell within the gonad primordium. *Sxr*-carrying females constructed in this way are fully fertile.

X/X^{Sxr} males (and T16H/X^{Sxr} males, which in the absence of banded karyotyping cannot be distinguished from X/X^{Sxr} males) are positive for H-Y transplantation antigen^{21,22}, showing that the translocated region contains the gene controlling H-Y,

whether or not this is identical with *Tdy*. We have shown recently that T16H/*X^{Sxr}* females also are positive for H-Y, whether tested by an *in vitro* cytotoxic T-cell assay or by the proliferative responses of H-Y-specific T-cell clones²³. If these *Sxr*-carrying females are indeed mosaics, containing a cell population in which the *Sxr* sequences are expressed, this result is not surprising. It is interesting that the presence of H-Y antigen in no way interferes with female reproduction.

However, one *Sxr*-carrying female out of 11 tested²³ consistently proved to be negative for H-Y antigen (female 39 in Table 1). This result could reflect one extreme of a mosaic distribution, in which virtually all cells have expression of the *Sxr* region suppressed by X-inactivation; such a situation would be a property of the individual and would not be inherited. Alternatively, it could reflect an alteration in the *Sxr* region, such that the masculinizing activity is retained but the antigenicity associated with the gene for H-Y is no longer expressed. If

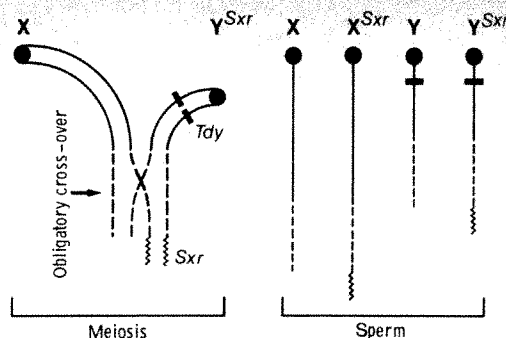


Fig. 1 Inheritance of *Sxr*. *Sxr* carrier males produce four different types of sperm, and hence progeny, because of the single obligatory cross-over between X and Y chromatids during male meiosis. The broken lines indicate pairing regions in the X and Y chromosomes.

Table 1 H-Y typing of mice shown in Fig. 2

Expt	Sex	Source of stimulating target cells (serial no.)	Genotype	Cytotoxicity				Proliferation (c.p.m. $\times 10^{-2}$)				Summary of typing	
				Anti-H-2 ^k	Anti-H-2 ^b	Anti-H-Y ^k	Anti-H-Y ^b	C-3/A-4 (D ^k +H-Y)	Clone (antigen specificity): 10-2-12 (D ^b +H-Y)	2-1-1 (I-A ^b +H-Y)	No. 4 (I-E ^k)	H-2	H-Y
1	♀	(39)	T16H/ <i>X^{Sxr}</i>	31	9	1	2	5	16	16	ND	k	-
	♀	(30)	XX	21	2	1	1	5	15	2	ND	k	-
	♂	(46)	XY	22	-1	17	-1	121	ND	1	ND	k	+
2	♂	(59)	(T16H or X)/ <i>X^{Sxr}</i>	16	15	1	4	31	71	4	ND	k/b	-
	♂	(58)	T16H/Y	16	19	29	22	539	1,290	38	ND	k/b	+
	♀	(55)	XX	11	0	0	0	45	52	9	ND	k	-
	♀	(64)	XX	ND	ND	ND	ND	99	70	10	ND	k	-
	♀	(68)	T16H/ <i>X^{Sxr}</i>	ND	ND	ND	ND	1,122	43	17	ND	k	+
3	♀	(69)	T16H/ <i>X^{Sxr}</i>	ND	ND	ND	ND	813	16	16	ND	k	+
	♂	(71)	T16H/Y	ND	ND	ND	ND	1,245	1,358	346	ND	k/b	+
	♂	(72)	T16H/Y	ND	ND	ND	ND	1,271	60*	163	ND	k/b	+
	♂	(73)	XY	ND	ND	ND	ND	1,009	1,645	677	ND	k/b	+
	♂	(74)	XY	ND	ND	ND	ND	1,261	1,367	265	ND	k/b	+
	♂	(117)	(T16H or X)/ <i>X^{Sxr}</i>	ND	ND	ND	ND	ND	20	2	ND	k/b	-
4	♂	(114)	T16H/Y	ND	ND	ND	ND	ND	760	90	ND	k/b	+
	♀	(121)	XX	ND	ND	ND	ND	ND	6	1	ND	k/b	-
	♂	(146)	(T16H or X)/ <i>X^{Sxr}</i>	40	TF	3	8	ND	ND	1	189	k	-
5	♂	(CBA)	XY	49	TF	39	8	ND	ND	4	233	k	+
	♀	(CBA)	XX	30	TF	0	9	ND	ND	15	224	k	-
6	♀	(197)	T16H/ <i>X^{Sxr}</i>	41	3	11	7	5	ND	4	590	k	-
	♂	(CBA)	XY	40	0	36	0	449	ND	3	334	k	+
	♀	(CBA)	XX	40	1	7	3	6	ND	4	419	k	-

Spleen cells were obtained by partial splenectomy from number-coded mice. The code was broken only after the results had been computed. To prepare stimulator cells for the T-cell clones, or targets for cytotoxic T cells or antibody plus complement lysis, red blood cells were removed from the spleen cell suspensions, which were resuspended at 8×10^5 ml in RPMI 1640 containing 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, penicillin 50 IU ml⁻¹ and streptomycin 50 µg ml⁻¹ (RPMI medium). Appropriate aliquots were taken for use as Con A blast targets (see below) and the cells to be used to stimulate the T-cell clones were irradiated (2,000 R) before being dispensed in volumes of 0.1 ml per well into flat-bottomed microtitre plates for the proliferation assay (see below). H-2 typing was performed with cytotoxic T cells (cell-mediated lysis, see below), and in experiments (5) and (6), with a proliferative T-cell clone specific for the I-E^k molecule. Typing was confirmed in experiments (2)-(6) using antibody plus rabbit complement with ⁵¹Cr-labelled splenic lymphocytes (either fresh, 2-day or 5-day Con A blasts) and monoclonal antibodies 28-13-3S (anti-K^b) and 12-2-2S (anti-K^d), each at a 1:7,500 dilution of ascites fluid (provided by Dr D. Sachs). A single-step cytotoxicity test was done in a final volume of 200 µl (50 µl cells, 50 µl antibody plus 100 µl rabbit complement at a final concentration of 1:30). Cell-mediated lysis (CML) was performed essentially as described in ref. 23 using as attacker cells 5-day mixed lymphocyte cultures (MLC) of the following type: anti-H-Y^b = C57BL/10 female immunized *in vivo* and boosted in MLC with C57BL/10 male spleen cells; anti-H-2^k = BALB.K or CBA anti-C57BL/10; anti-H-Y^k = (C57BL/10 $\times$ CBA)F₁ female immunized *in vivo* and boosted *in vitro* with CBA male cells; anti-H-2^k = C57BL/10 or BALB.B anti-CBA. Target cells were splenic lymphocytes held in culture before the CML assay either for 2 days with RPMI medium containing 4 µg ml⁻¹ Con A or for 5 days in RPMI medium with 1 µg ml⁻¹ Con A, and 25% rat spleen Con A supernatant as a source of interleukin-2 (IL-2). 2-day targets (in 2-ml volumes) were cultured in cluster plate wells at 5×10^5 ml⁻¹; for 5-day targets, 1×10^6 cells were cultured in 12.5-ml volumes in 50-ml tissue culture flasks kept upright. Target cells were labelled with ⁵¹Cr and 10⁴ cells per well were added in 100-µl volumes to round-bottomed microtitre wells in plates into which attacker cells had been titrated (100 µl volumes) in triplicate in four threefold dilutions, giving a starting (highest) attacker:target (A:T) cell ratio of ~30:1. Controls consisted of target cells incubated with 100 µl medium alone (medium control, C) and target cells with 100 µl 5% Triton X-100 (maximum release, M). After incubation for 3 h, the assay plates were centrifuged briefly, 100 µl of supernatant collected for γ counting. Per cent specific lysis was calculated as $E - C/M - C$ where E = c.p.m. of target cells incubated with attacker cells, and C and M are the c.p.m. of target cells incubated with medium alone and with Triton X-100, respectively. Per cent lysis was calculated for each A:T ratio on each target, and regression analysis was performed for each attacker/target combination. The per cent lysis at A:T = 10:1 was computed from the regression curves; this value is given in the table. Positive values are defined as those obtained from titration data, with a correlation coefficient $r^2 \geq 0.80$; these values are italicized. Proliferative clones: C-3 and 10-2-12 have been used previously for H-Y typing²³, and are specific for D^k+H-Y and D^b+H-Y, respectively. These clones are IL-2-dependent, therefore IL-2 (rat spleen Con A supernatant) was included in the assays at 10-20%. Clones 10-2-12 and 2-1-1 have been described previously²⁸. Clone 2-1-1 is specific for I-A^b+H-Y and is IL-2 independent. Clone A-4, like C-3, recognizes D^k+H-Y and is IL-2-dependent (K.T., unpublished). Clone no. 4 recognizes the I-E^k molecule and is IL-2-independent (K.T., unpublished). Cells from these clones were used in the proliferation assays 12-14 days after their last *in vitro* restimulation. They were plated at 10⁴ per cells per well together with either 8×10^3 stimulator cells per cell or medium alone. After 3 days of culture, clones 2-1-1 and no. 4 were pulsed with ³H-thymidine for 6 h; after 4 or 5 days of culture clones 10-2-12, C-3 and A-4 were similarly pulsed. DNA-bound thymidine was collected onto filters for β-counting. The results shown are an average of triplicate wells. Clone C-3 was used in experiments (1), (2) and (3), whereas clone A-4 was used in experiment (6), as it was growing better at the time. Insufficient C-3 cells were available for use in experiments (4) and (5). Background counts of cloned cells incubated in medium (plus IL-2 in the case of the IL-2-dependent clones C-3, A-4 and 10-2-12) without stimulator cells were <1,000 c.p.m. (normally <500 c.p.m.) in all experiments except (2) in which they were 2,372 for C-3. Counts of cloned cells incubated with female stimulator cells were sometimes higher than medium $\pm$ IL-2 alone and this may reflect 'feeder effects' $\pm$ back stimulation, since the clones in some cases expressed an H-2 type not present on the stimulator cells. Where the c.p.m. in the presence of female stimulator cells was high (as in expts (2) and (3) for clones C-3 and 10-2-12), that with male cells was very high indeed (more than an order of magnitude higher), so that discrimination between positive and negative was clear. The data shown for each experiment are restricted to mice shown in Fig. 1, plus additional controls where necessary to evaluate the data. However, each H-2 and H-Y typing experiment included generally at least 20 mice (for example, the data in expt (1) are taken from a much larger experiment published in full in ref. 23). To date, we have typed over 200 mice in this study; for all mice other than 39, 59, 117, 146 and 197 (all H-Y-negative although each carries *Sxr*) the H-Y typing with T-cell clones and CML was concordant with the genotype, that is, all males with a Y chromosome or *X^{Sxr}* were H-Y-positive, as were T16H/*X^{Sxr}* females. ND, not done. TF, technical failure: the anti-H-2^b attacker cells used in this experiment failed to lyse control C57BL/10 male and female targets.

* From the data using clone 10-2-12, mouse 72 is a presumed intra-H-2 recombinant and lacks the D^b molecule; serological typing shows that it expresses the K^b molecule, and the results with clone 2-1-1 show that it expresses the I-A^b molecule and is H-Y-positive. Clone C-3 confirms that mouse 72 is H-Y-positive and expresses D^k.

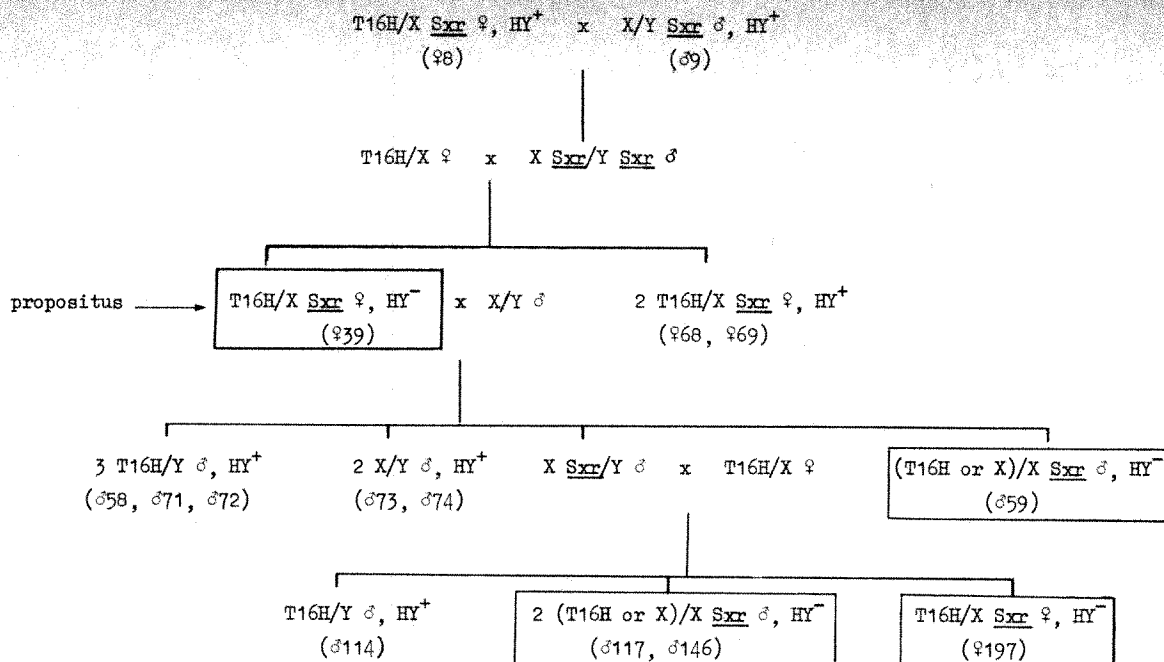


Fig. 2 Family tree of *Sxr*-carrying ♀39 (see Table 2 of Simpson *et al.*²³). The stocks of mice used and the production of T16H/*X^{Sxr}* females are described by McLaren and Monk¹⁹. T16H/*X^{Sxr}* females, *X/Y^{Sxr}* males and *X^{Sxr}Y^{Sxr}* males were identified by progeny testing. T16H/*Y* and *X/X^{Sxr}* males were distinguished from *X/Y* males and from each other by testis weights, combined where necessary with testis histology and often also with karyotyping. T16H/*X^{Sxr}* males were not distinguished from *X/X^{Sxr}* males. Only those mice tested for H-Y antigen have serial numbers. For details of H-Y antigen testing, see Table 1.

the alteration is stable, it should be detected in subsequent generations.

Table 1 shows the results of testing female 39 herself, one (T16H or X)/*X^{Sxr}* son, two (T16H or X)/*X^{Sxr}* grandsons and one T16H/*X^{Sxr}* granddaughter, together with appropriate control mice, using both cytotoxic T cells and H-Y-specific T-cell clones. In experiments (1), (2) and (6), H-2 type and H-Y type were assigned on the basis of the cytotoxicity assay, and independently confirmed by the clonal proliferation test. In experiments (3) and (4), H-2 and H-Y were assigned on the clonal test alone; in experiment (5), H-Y type was assigned by the cytotoxicity results, but the clonal test confirmed the H-2 typing. In addition, H-2 typing by monoclonal antibody plus complement was used to confirm the T-cell H-2 typing in each experiment except (1).

Details of the cytotoxic T-cell, clonal proliferation and antibody plus complement lysis assays are given in Table 1. Briefly, spleen cell suspensions were made from partial splenectomy samples of each mouse to be tested. After removal of red blood cells, the cell suspension was divided into two aliquots, one of which was placed in culture with concanavalin A (Con A) to generate blast cells for use as target cells 2 or 5 days later in both the cytotoxic T cell and antibody plus complement lysis assays. The other aliquot was irradiated and used to stimulate the T-cell clones. All the evidence is consistent with the H-Y antigen defined by cytotoxic T cells and H-Y-specific T-cell clones being the same as that defined by transplantation.

The family tree of ♀39 is shown in Fig. 2; it is apparent that all tested descendants of ♀39 who have inherited her mate's Y chromosome are, as expected, positive for H-Y antigen; on the other hand, those who have received her *Sxr* region in the absence of a Y chromosome, that is, *X/X^{Sxr}* males and T16H/*X^{Sxr}* individuals (whether male or female), are H-Y-negative. Histological examination of the testes of the three H-Y-negative (T16H or X)/*X^{Sxr}* males suggests that they do not differ from those of H-Y-positive (T16H or X)/*X^{Sxr}* males. It seems, therefore, that we are dealing with a variant form of the *Sxr* region, which retains the testis-determining property but has lost the sequences controlling the expression of H-Y trans-

plantation antigen. We propose that this variant be designated *Sxr'*, with the constitution *Tdy⁺, Hya⁻*, where *Hya* is the locus (structural or regulatory) controlling the expression of H-Y antigen. *Tdy* and *Hya* may be the same locus, or two separate loci.

Our data do not allow us to determine whether *Sxr'* lacks the *Hya* locus altogether, or retains it in a modified form such that the antigenicity by which the molecule is defined has been lost. In either case, the separation of testis determination from expression of H-Y transplantation antigen eliminates this antigenic determinant as a candidate for the primary sex determinant. Previous reports point to the same conclusion. A single sterile XO male described by Melvold *et al.*²⁴ had testes but was negative for H-Y antigen; while many H-Y-positive females have been described, not only the presumed mosaic T16H/*X^{Sxr}* females mentioned above²³, but also several XY females reported by Eicher and colleagues^{25,26}.

If mice lacking the H-Y transplantation antigen can develop as phenotypic males, what is the function of the H-Y antigen in normal males? Such a ubiquitous substance, present as far as we know on all cells of all male mice examined, except for those described here and the single male described by Melvold and colleagues²⁴, should be of fundamental significance. We are examining the possibility that H-Y is required for some aspect of male reproduction, for example spermatogenesis. Any such defect would not manifest itself in our H-Y-negative males, as they are sterile, with germ cells that degenerate soon after birth and do not undergo spermatogenesis, because of the presence of two X chromosomes.

We do not know whether the event giving rise to the variant *Sxr'* region occurred in the cell lineage of the spermatozoon contributing to ♀39, or in a previous generation. However, it may be no coincidence that ♀39 was the daughter of a homozygous *X^{Sxr}Y^{Sxr}* male (Fig. 2), because the presence of two homologous *Sxr* regions, containing copious repeated DNA sequences²⁷, would be expected to facilitate unequal crossing-over and hence separation of *Hya* and *Tdy*, if they are indeed two separate loci, or loss of part of *Hya*. We have so far examined 65 progeny of homozygous and 99 progeny of heterozygous *Sxr*

males, without detecting another such event. The unequal crossing-over model makes no prediction as to the order of *Hya* and *Tdy* within the *Sxr* region, but requires that the *Hya* locus is flanked on both sides by homologous sequences.

Note added in proof: We have continued the pedigree shown in Fig. 2 to five generations ($\varnothing 39$ = generation 0). Of 19 (T16H or X)/X^{Sxr} males and 2 T16H/X^{Sxr} females typed so far in this pedigree, all have proved HY negative.

Received 11 June; accepted 14 September 1984.

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Analysis of persistent virus infections by *in situ* hybridization to whole-mouse sections

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Nucleic acid hybridization techniques have contributed significantly to the understanding of gene organization, regulation and expression¹⁻⁴. In the context of persistent or latent viral infections, hybridization with specific labelled probes represents the most sensitive assay presently available for detection of viral genomes⁵⁻⁸. During the course of persistence, viral genomes may exist in multiple and yet quite segregated areas in an infected host, but examination of all tissues remains difficult and time-consuming. At present, the application of hybridization assays to *in vivo* infections requires either chemical extraction of nucleic acid coupled with dot-blot⁹ and gel transfer¹ techniques or *in situ* hybridization^{5,10} to cryostat tissue sections. In both cases, selected tissues must be removed by dissection before analysis. We describe here a procedure which allows efficient and reproducible screening of all tissue in an infected host. Our technique allows detection of viral genetic material in whole-body sections of infected mice, and provides the first evidence *in vivo* for accumulation of viral genetic material with a parallel decrease in infectious virus during persistent virus infection. This technique should be widely applicable to studies of developmental regulation of gene expression, for monitoring locations of gene expression in transgenic mice and for analysis of molecular mechanisms in pathogenesis.

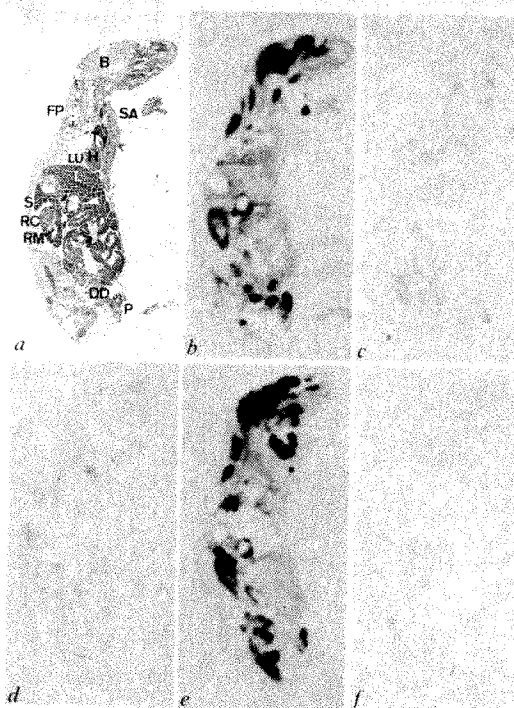


Fig. 1 *In situ* hybridization to whole-body sections of a persistently infected SWR/J mouse and age- and sex-matched controls. *a, b, f*, Sections from a persistently infected 12-week-old mouse; *a* and *b* are different representations of the same 40-μm section. Histopathological details are recorded in *a* and relate to: B, brain; SA, salivary gland; FP, brown fat pad; T, thymus; H, heart; LU, lung; L, liver; S, spleen; RC, renal cortex; RM, renal medulla; DD, ductus deferens; P, penis. The arrow indicates the mucosa of the stomach. *b* Shows the corresponding pattern of tissue hybridization with a cloned cDNA probe derived from the S RNA segment of LCMV: Armstrong CA 1371. *c*, Hybridization of the LCMV S cDNA probe to an uninfected control mouse. *d, e*, Adjacent 40-μm sections from a 10-week-old infected mouse; *d*, hybridization with a cytomegalovirus probe from the immediate-early region²⁵; *e*, hybridization with the cloned LCMV S cDNA probe. *f*, The effect of treating a section with RNase before hybridization with the LCMV probe. *In situ* hybridization to SWR/J mice is also shown in Fig. 3 where a moderate hybridization signal is seen for the livers; this level of signal is typical of SWR/J liver tissue and the liver signal in Fig. 1 is unusually low.

Methods: Mice were killed with ether, exsanguinated, shaved and then frozen in blocks of 2.5% carboxymethyl cellulose (CMC) by slow immersion in dry ice ethanol. These blocks could be stored at -30°C for several weeks with no apparent effect on signal intensities. The CMC blocks were rapidly cut to the relevant planes on an LKB cryomicrotome, then sequential 40-μm sections were collected on Scotch tape. With the tape firmly pressed against the CMC block, the attached section could be lifted away as the block was moved under the microtome blade. The sections were air-dried, fixed by immersion in freshly prepared 4% paraformaldehyde (in phosphate-buffered saline, PBS), rinsed twice in PBS and again air-dried. Pairs of sections were usually hybridized in Seal-A-Meal bags. Wetting the tape allows easy transfer of the sections to the bags. Prehybridization was performed at 37°C for 12-24 h in 50% deionized formamide, 5× SSC, 2.5× Denhardt's solution with 100 μg ml⁻¹ boiled, sonicated salmon sperm carrier DNA; 6 ml of solution were used for each pair of sections. Hybridization was performed at 37°C for 60-68 h using 2 ml of the same solution for each pair of sections and 2-4 × 10⁶ c.p.m. of ³²P-labelled nick-translated DNA probe, specific activity 1-5 × 10⁸ c.p.m. per μg DNA. In all cases, the LCMV cDNA inserts were excised from plasmid vectors and purified by gel electrophoresis before labelling. The average fragment length of the denatured probe was 200-400 bases. The sections were washed three times for 30 min each at 37°C in 2× SSC, and once for 30 min at 55°C in 2× SSC. After a final 30 min wash at 55°C in 0.1× SSC, the sections were stained with haematoxylin-eosin (modified to substitute lithium carbonate for the ammonium carbonate wash) and exposed at -70°C using Kodak XAR-5 film and a Cronex Lightning Fast intensification screen. The film was developed after 20 h exposure.

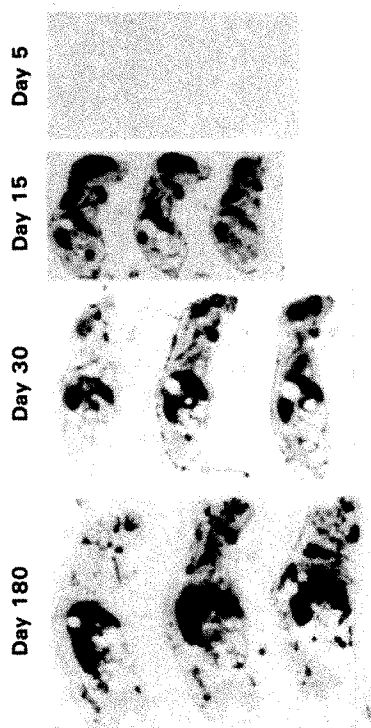


Fig. 2 BALB/WEHI mice infected at birth with LCMV (60 PFU given intracerebrally) were killed at the indicated times. Whole-body sections from individual mice were hybridized with a cloned cDNA probe derived from part of the LCMV S genomic RNA segment (see Fig. 1 legend and ref. 17 for details). This study shows *in situ* hybridization with sections from different animals at different time points (four mice at day 5, three mice at days 15, 30 and 180). Data representative of 17 mice studied. Hybridization to the skin (visible especially with the day 30 animals) is not normally seen.

Inoculation of newborn mice with lymphocytic choriomeningitis virus (LCMV) readily leads to persistent virus infection¹¹⁻¹⁴. Following infection, mice of most inbred strains reach maturity but retain life-long viraemia, with infectious virus present in blood and major organs. The genome of LCMV comprises two single-stranded RNA segments of negative polarity (RNAs designated L and S, approximate lengths 9 and 4 kilobases (kb), respectively) that exist as ribonuclear protein complexes within infected cells and virions^{15,16}. We have prepared and characterized partial complementary DNA clones to both the L and S genomic RNA segments¹⁷. These clones have been labelled with ³²P by nick-translation¹⁸ for use as hybridization probes¹⁹.

Typical results from *in situ* hybridization are shown in Fig. 1, where the contrast between positive and negative controls is clear. The specificity of the hybridization reaction was verified by three independent approaches in several experiments. There was no positive signal from sections of uninfected, age- and sex-matched control animals that were hybridized with the LCMV probes (Fig. 1c) and pretreatment of LCMV-infected whole-mouse sections with RNase effectively abolished the hybridization signal (Fig. 1f). Sections from LCMV-infected animals that were hybridized with a probe from an unrelated virus (human cytomegalovirus) also failed to show any signal (Fig. 1d). There was good tissue preservation during the experimental manipulations and, after hybridization, sections could be treated with histological stains (Fig. 1a, b) before autoradiographic exposure to facilitate tissue identification. Obviously, any single section may not include all tissue types, but serial sections were made at different depths throughout the animal

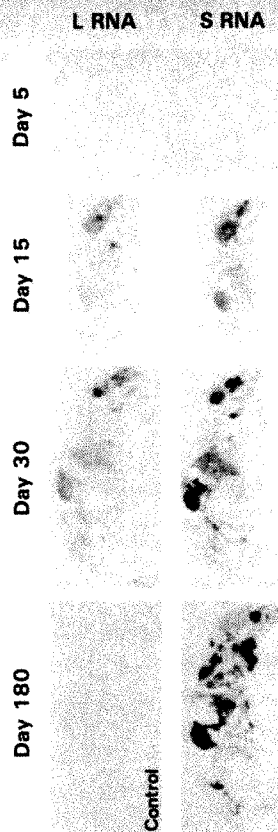


Fig. 3 SWR/J mice infected at birth with LCMV (60 PFU given intracerebrally) were killed at various ages. Whole-body sections from individual mice were hybridized with cloned LCMV cDNA probes to part of either the large (L) RNA or small (S) genomic RNA segments. Two mice are shown for day 5. The control is an uninfected mouse section reacted with the ³²P-labelled L RNA probe. This study shows similar distribution of L and S genetic material in different tissues during infection (the distribution of L RNA in tissues from the 180-day-old mouse was similar to that shown with the S probe). The data are representative of >20 individual SWR/J mice studied.

to ensure sampling of all the major tissues at various planes. This whole-body sectioning revealed viral genetic material in unexpected locations, for example, the mucosa of the stomach (Fig. 1, arrowhead) and the ductus deferens (Fig. 1, DD). Other experiments using a cDNA probe derived from 28S ribosomal RNA (P.J.S., unpublished results) showed positive hybridization to many tissues, with the strongest signal exhibited by thymus (data not shown).

During these studies we compared several support substances, tissue fixatives and detergent treatments in an attempt to optimize the hybridization procedure. We found that picking up tissue on 3M Scotch tape was preferable to using Gene Screen Plus (NEN), nitrocellulose (Schleicher & Schuell) or a glass slide. Gene Screen Plus was similar in signal-to-noise ratio and tissue retention to 3M tape, but staining with haematoxylin and eosin for morphological details was unsatisfactory. Hybridization to sections on nitrocellulose showed a 5-10-fold signal reduction and, after baking at 80°C, there was an increased tendency to tissue loss during the hybridization procedure. Sections picked up on glass slides varied in terms of tissue retention. Multiple procedures using proteases and Nonidet-P40 showed no significant enhancement of the hybridization signal. We usually cut 40-µm sections for hybridization, but some were as thin as 15-µm. Once cut and fixed, the sections could be stored dry for up to 3 weeks with no loss of hybridization signal. Of various fixatives we have tried, 4% paraformaldehyde seemed the most effective.

Figure 2 illustrates the distribution of viral genetic material during the lifespan of persistently infected BALB/WEHI mice. The patterns of hybridization are highly reproducible for

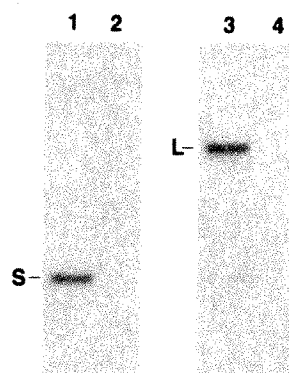


Fig. 4 Total cellular RNA from LCMV-infected or uninfected L-929 cells was extracted by the guanidinium thiocyanate procedure²⁶, denatured with glyoxal, separated by agarose gel electrophoresis, then transferred to a nitrocellulose filter²⁷. The filter was hybridized with ³²P-labelled probes as described for the mouse section hybridization experiments. Lane 1, RNA (3 µg) from L-929 cells persistently infected with LCMV. Lane 2, RNA (3 µg) from a parallel culture of uninfected L-929 cells; the probe was an S-specific cDNA clone. Lanes 3, 4, the same nitrocellulose filter after stringent washing and rehybridization with an L-specific cDNA clone. The apparent hybridization of the L probe to an 'S-sized' RNA species is being investigated.

individual mice at each different time. Viral RNA was readily detectable in sections made 15 days after birth but sections made 5 days after birth showed a barely detectable hybridization signal, although infectious virus can be isolated from tissues and blood of infected animals at this time^{13,14}. With increasing age, there was no consistent change in the distribution of viral RNA when comparing persistently infected BALB mice at 15, 30 or 180 days. The titre of infectious LCMV recovered from the brains of individual BALB/WEHI mice 5 days after initiating infection in plaque-forming units (PFU) per g (mean for five mice: 6×10^6 , range 7×10^5 – 9×10^6) was over 2,000-fold higher than that obtained from mice at 180 days (mean for five mice: 3.0×10^3 , range 1×10^3 – 7×10^3), yet the accumulation of viral nucleic acid sequences was marked at 180 days and insignificant at 5 days. The virus titre at 15 and 30 days after infection was 1.4×10^5 and 2.5×10^4 , respectively (values represent the mean for five animals). In all cases, virus titres were measured in plaque assays using Vero cell monolayers in standard conditions¹⁴. These experiments showing increased levels of viral nucleic acid accompanied by a significant reduction in the titre of infectious virus in brain tissue, were duplicated over the course of persistent infection in a different strain of mouse, SWR/J (see Fig. 3). The titre of infectious LCMV (PFU per g) recovered from the brains of individual SWR/J mice 5 days after initiating infection was 2.2×10^7 (mean for four animals, range 2×10^6 – 4×10^7) and at 180 days the titre was 2.5×10^4 (mean for five animals, range 7×10^3 – 8×10^4). The apparent discrepancy between increased levels of viral nucleic acid and reduced titres of infectious virus suggests the presence of defective particles generated during the course of natural infection, a possibility that we are investigating. This theoretical possibility has been raised by others^{20,21} primarily on the basis of experiments with cultured cells.

Comparison of sections from persistently infected BALB/WEHI mice (Fig. 2) and SWR/J mice (Figs 1, 3) indicated that there was a significant difference in the levels of LCMV nucleic acid sequences in the liver whereas other tissues were similar in both mouse strains. For BALB/WEHI mice, 15 of 17 animals showed strong hybridization to liver whereas equivalent strong hybridization was seen in only 3 of 20 SWR/J mice.



Fig. 5 Whole-body *in situ* hybridization and histopathological analysis of a single section taken from a persistently infected, pregnant BALB/WEHI mouse 19–20 days after conception. This female mouse had been mated with a persistently infected BALB/WEHI male. *In situ* hybridization was performed as described for Fig. 1, using a cloned cDNA probe derived from part of the LCMV L RNA. Two embryos can be observed in the stained section. There is a strong hybridization signal from the liver (L) and brain (B) of the pregnant mouse. The area of strong hybridization above the thymus in the pregnant mouse is from the salivary gland. H, heart; E1, embryo 1; E2 embryo 2. The hybridization pattern to embryo 1 is highly suggestive of a central nervous system location for viral genetic material and this is verified by the accompanying histopathology. Similar results were obtained with two additional congenitally infected pregnant mice using an LCMV S-specific probe.

In the next series of experiments, we analysed adjacent 40-µm sections from SWR/J mice during the course of persistent infection, using equivalent probes derived from the L and S genomic segments of LCMV (Fig. 3). There was no difference in the tissue patterns of hybridization for the L and S probes. The signal from L probes was normally about half that from S probes, consistent with our analysis of total RNA extracted from LCMV-infected tissue culture cells (data not shown). Figure 4 shows the specificity of the L and S RNA probes.

The sensitivity of the *in situ* hybridization technique has been assessed by dot-blot experiments⁹ using cloned LCMV cDNAs as standards. We have excised selected tissue areas from 40-µm sections and extracted total nucleic acid by SDS/proteinase K and phenol treatments to provide a direct measure of the LCMV RNA content in the section. For example, by hybridization with an S-specific cDNA probe, we estimate that there are ~250 pg of 'S-derived' RNA in the kidney sections of 30-day-old BALB/WEHI and SWR/J mice (Figs 2, 3). On this basis and together with data from other tissues, we calculate that the sensitivity of hybridization to tissue sections is reduced 2–10-fold relative to the dot-blot method. We are currently investigating several parameters to increase the sensitivity of the technique by enhancing the specific activity of the probes using strand-specific probes from M13 (ref. 22) or SP6 (ref. 23) vectors. Results reported here were obtained with ³²P probes of specific activity 1 – 5×10^8 c.p.m. µg⁻¹.

Whole-body *in situ* hybridization proved useful in studying the tissues of persistently infected pregnant mice. Figure 5 shows viral genetic material in congenitally infected embryos of a pregnant, persistently infected BALB/WEHI mouse. One embryo (Fig. 5, E1) shows a heavier load of viral RNA than the other (Fig. 5, E2). Studies of other congenitally infected pregnant mice revealed similar uneven accumulation of viral nucleic acid sequences among the carrier embryos. We are currently evaluating the expression of LCMV genetic material in developing embryos, from gestation until after delivery, and the expression of developmentally regulated genes like those associated with myelination.

Thus, whole-animal sections can now be studied by *in situ* hybridization and locations and changes in viral gene expression can be mapped. Several limitations of the conventional *in situ*

hybridization procedure are largely avoided. There is good preservation of tissue and sections can be treated with histological stains after the hybridization procedure. Concurrent with these studies, Dubensky *et al.*²⁴ have recently detected DNA and RNA in organs of mice following polyoma and vesicular stomatitis virus infections. Finally, preliminary results indicate that LCMV antigens can be detected in mouse sections using immunological reagents. Hence, it is now possible to detect expression of viral RNA and protein while maintaining suitable morphology for detailed study at the whole-animal level.

Received 25 June; accepted 20 September 1984.

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We thank Drs Ashley Haase, Luis Villarreal and Michael Wilson for their comments and suggestions and Ms Gay L. Wilkins for secretarial assistance. This work was supported in part by USPHS grants NS-12428, AG-04342 and AI-09484 and by USAMRIID contract C-3013. This is publication 3500-IMM from the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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Serine- and threonine-specific protein kinase activities of purified gag-mil and gag-raf proteins

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Retroviruses carry cell-derived oncogenes (*v-onc*) that have the potential to transform cells in culture and induce tumours *in vivo*^{1,2}. One of the few carcinoma-inducing viruses is the acutely transforming retrovirus MH2 (refs 2, 3), which carries the putative oncogene *v-mil* and the known oncogene *v-myc* (refs 4-6). Recently, a high degree of homology was discovered between *v-mil* and *v-raf* (ref. 7), the transforming gene of the murine retrovirus 3611 murine sarcoma virus (MSV)⁸, whereas homology to *v-src* is low⁹. Both viruses express their oncogenes as the gag-fusion polypeptides^{6,10} p100^{gag-mil} and p75^{gag-raf} (of respective relative molecular mass (*M_r*) 100,000 and 75,000), while the *myc* oncogene of MH2 is expressed by means of a subgenomic messenger RNA¹¹. We have recently demonstrated that p100^{gag-mil} is not a nuclear protein⁶. Here we report that purified p100^{gag-mil} and p75^{gag-raf} exhibit protein kinase activities *in vitro* which, in contrast to the *src*-related p130^{gag-fps} of Fujinami sarcoma virus (FSV)¹² and all other characterized oncogene-encoded protein kinases, phosphorylate serine and threonine but not tyrosine. Both types of protein kinases phosphorylate lipids *in vitro*.

Previously reported protein kinase studies of p75^{gag-raf} have involved immunoprecipitation analyses. We have recently been able to recover highly purified and enzymatically active p130^{gag-fps} protein kinase from FSV-transformed cells¹³⁻¹⁶ (P. Donner *et al.*, manuscript in preparation), and have applied our protocol here to investigate whether p100^{gag-mil} and p75^{gag-raf} possess protein kinase activity. Immunoaffinity purification of p100^{gag-mil} and p130^{gag-fps} was performed by using monoclonal immunoglobulins (IgG) against p19, the amino-terminus of the gag portion¹⁷. ³⁵S-methionine-labelled cellular extracts of FSV- and MH2-transformed cells were applied to the columns to purify p100^{gag-mil} and p130^{gag-fps} (Fig. 1). As a control, p110^{gag-myc} from MC29-transformed quail fibroblasts was also purified

(p110, Fig. 1). Purification efficiency by this procedure is about 3,000-fold¹⁷.

p75^{gag-raf} carries a murine viral gag protein and cannot be isolated using the column described above. Therefore, IgG specific for the murine viral structural protein p30 was purified from polyvalent anti-p30 rabbit serum and used to set up an immunoaffinity column. p75^{gag-raf} was isolated from ³⁵S-methionine-labelled 3611 MSV-transformed rat fibroblasts. 3611 MSV codes for a doublet consisting of a glycoprotein of *M_r* 90,000 (gp90^{gag-raf}) and p75^{gag-raf} (ref. 8). Figure 1 shows the purified proteins. Two additional bands were detected which have been observed previously in immunoprecipitates and are considered to be gag-processing proteins¹⁰.

Both types of immunoaffinity columns were treated with 0.5% SDS after elution of the ³⁵S-methionine-labelled gag-fusion proteins so as to isolate possible residual proteins remaining on the immunobeads. No such proteins were detected (data not shown). Similarly, when aliquots of the immunoaffinity columns loaded with the ³⁵S-methionine-labelled gag-fusion proteins were analysed by polyacrylamide gel electrophoresis in analogy to the protein kinase experiments described below, no protein contamination was detected (data not shown).

The proteins recovered from ³⁵S-methionine-labelled cellular extracts showed no protein kinase activity. Enzymatically active proteins were recovered only from unlabelled cellular lysates applied to the immunoaffinity columns using a modified approach. Instead of being eluted from the columns, the proteins were allowed to remain attached to the immunobeads for protein kinase assay. The assays used 10 mM Mg²⁺ and 10 mM Mn²⁺ as divalent cations (Fig. 2); the optimum ion concentration has been determined to be 10 mM Mn²⁺ in all cases (data not shown). Autophosphorylation of p100^{gag-mil}, p75^{gag-raf}, and p130^{gag-fps} was detected, but no autophosphorylation occurred with gp90^{gag-raf} or p110^{gag-myc}. In the case of 3611, two additional bands of lower relative molecular mass were phosphorylated. Preliminary evidence indicates that these bands are the result of proteolytic processing¹⁰. The absence of protein kinase from MC29-transformed quail fibroblasts after even longer exposure times indicates that the p100^{gag-mil} protein kinase activity is not of normal quail cell origin.

An additional control experiment was performed to exclude the possibility that the serine/threonine protein kinase was of rat cell origin. 3611 MSV cells were passed over an anti-p19 column as a nonspecific IgG-containing column. No protein kinase activity was detected (Fig. 2a, control). The autophosphorylation reactions were standardized to identical protein

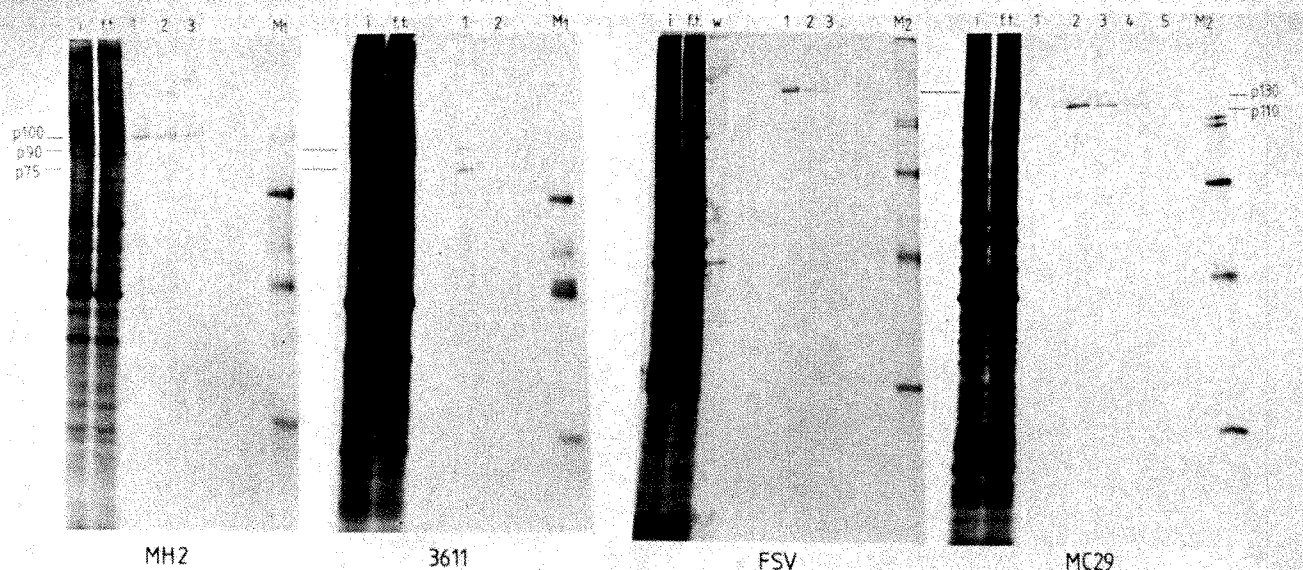
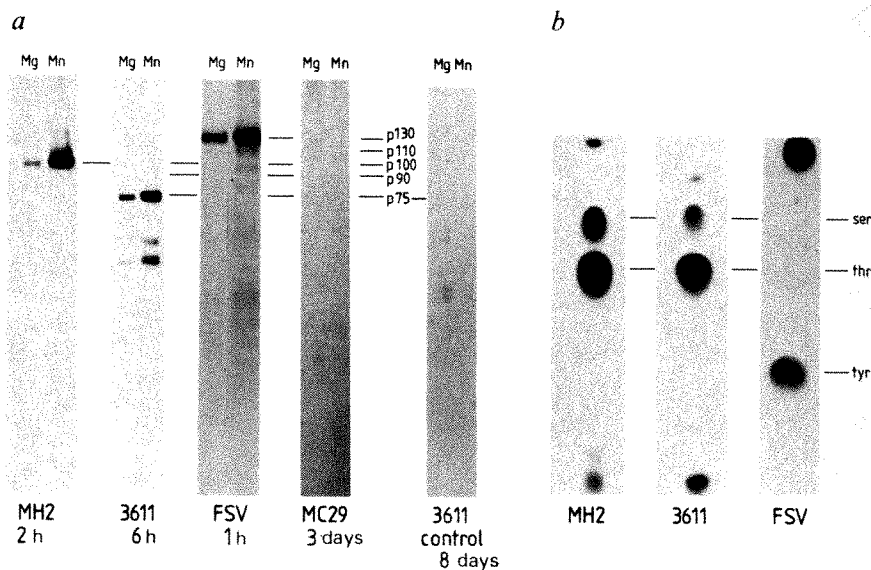


Fig. 1 Immunoprecipitation of p100^{gag-mil}, p75^{gag-raf}, p130^{gag-fps}, and p110^{gag-myc} proteins. i, Input; f.t. flow through; lanes 1-5, eluted fractions; M₁, M₂, size markers ($M_r \times 10^{-3}$) from top to bottom: M₁, 92, 69, 55, 46, 30; M₂, 92, 69, 46, 30. Specific activities of the eluted proteins were: p90/75^{gag-raf}, 8×10^4 c.p.m. μg^{-1} ; p130^{gag-fps}, 5×10^4 c.p.m. μg^{-1} ; p100^{gag-mil}, 3.6×10^4 c.p.m. μg^{-1} ; p110^{gag-myc}, 6×10^4 c.p.m. μg^{-1} . **Methods:** The cell lines used in this study were MH2-transformed non-producer quail fibroblasts⁶, 3611 MSV-transformed non-producer rat fibroblasts⁸, FSV-transformed rat fibroblasts¹² and MC29-transformed non-producer quail fibroblasts¹⁷. 5×10^7 cells of each of the cell types were labelled with ³⁵S-methionine (250 $\mu\text{Ci ml}^{-1}$) at 80% confluency for 2 h, lysed with 10 ml RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100), 10 U ml⁻¹ Trasylol, centrifuged (10,000 r.p.m., 30 min, 4°C) and applied to immunoaffinity columns containing IgG against the murine viral protein p30 in the case of 3611, and against p19 for the other cell types. The anti-p30 column was prepared as follows: p30 was isolated by phosphocellulose column chromatography from 50 mg of MSV using elution conditions described elsewhere²³. Isolated p30 (1 mg) was coupled to activated CH-Sepharose 4B (Pharmacia). Goat anti-p30 serum (10 ml) was passed over the p30 column and specific IgG recovered. This anti-p30 IgG (2 mg) was then coupled to protein A-Sepharose 4B (0.2 g) as described elsewhere¹⁷ and used as anti-p30 immunoaffinity column. The anti-p19 immunoaffinity column has been described elsewhere¹⁷. After extensive washing, the purified proteins were eluted with citric acid buffer, pH 3.5, which was neutralized immediately. The eluted proteins (30 μl per 2 ml of each fraction) were analysed by gel electrophoresis and autoradiography.

Fig. 2 a, Protein kinase assay of purified proteins. b, Phosphoamino acid analysis.

Methods: a, 2×10^7 cells of MH2-, 3611-, FSV- and MC29-transformed non-producer fibroblast lines each were lysed with RIPA buffer in the presence of 1 mM dithiothreitol, centrifuged (10,000 r.p.m., 30 min, 4°C) and applied to immunoaffinity columns (200 μl of packed beads each) as described in Fig. 1 legend. 3611 lysates were also applied to an anti-p19 immunoaffinity column (3611 control). The columns were washed extensively¹⁷. Instead of eluting the proteins from the columns, they remained immobilized on the beads and were distributed to reaction tubes for further analysis. Protein kinase activities were tested in a total volume of 50 μl containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, supplemented with 10 mM MgCl₂ (Mg) or 10 mM MnCl₂ (Mn). 5 μl enzyme-coupled beads were used per reaction. Incubation time was 10 min at 37°C. 20 μCi of [γ -³²P]ATP (3,000 Ci mmol⁻¹) were used per assay. Reactions were terminated by the addition of four-fold concentrated sample buffer¹⁷ for SDS-polyacrylamide gel electrophoretic analysis. Samples were treated for 1 min at 100°C and applied directly to the gels. The gels were dried and exposed for autoradiography for the indicated periods of time. b, The autophosphorylated proteins were cut out of the gels, eluted and processed for phosphoamino acid analysis as reported previously¹⁸. Exposure time was 3-5 days at -70°C using intensifier screens.



contents of the lysate inputs. The immunoaffinity columns contained excess IgG and bound all available gag-fusion proteins. Based on these findings, we determined the radioactivity incorporated into the individual bands and found that p130^{gag-fps} incorporated fivefold more radioactivity than did p100^{gag-mil} or p75^{gag-raf}.

The phosphoamino acid content of the autophosphorylated proteins was determined¹⁸. Figure 2b shows that p100^{gag-mil} and p75^{gag-raf} are not tyrosine-specific protein kinases like p130^{gag-fps}, but phosphorylate serine and threonine instead. As both

p130^{gag-fps} and p75^{gag-raf} originate from rat cells¹², the differences in amino acid phosphorylation rule out protein kinase contamination by rat cells.

p75^{gag-raf} lacks the tyrosine acceptor site found in src¹⁹. It was therefore important to analyse the amino acid specificities of p100^{gag-mil} and p75^{gag-raf} not only in autophosphorylation reactions but also with exogenous substrates. For this we used rabbit muscle actin, calf thymus arginine- and lysine-rich histones, chicken H5, and phosphotitin; casein was not used because of its high degree of autophosphorylation. The three protein kinases

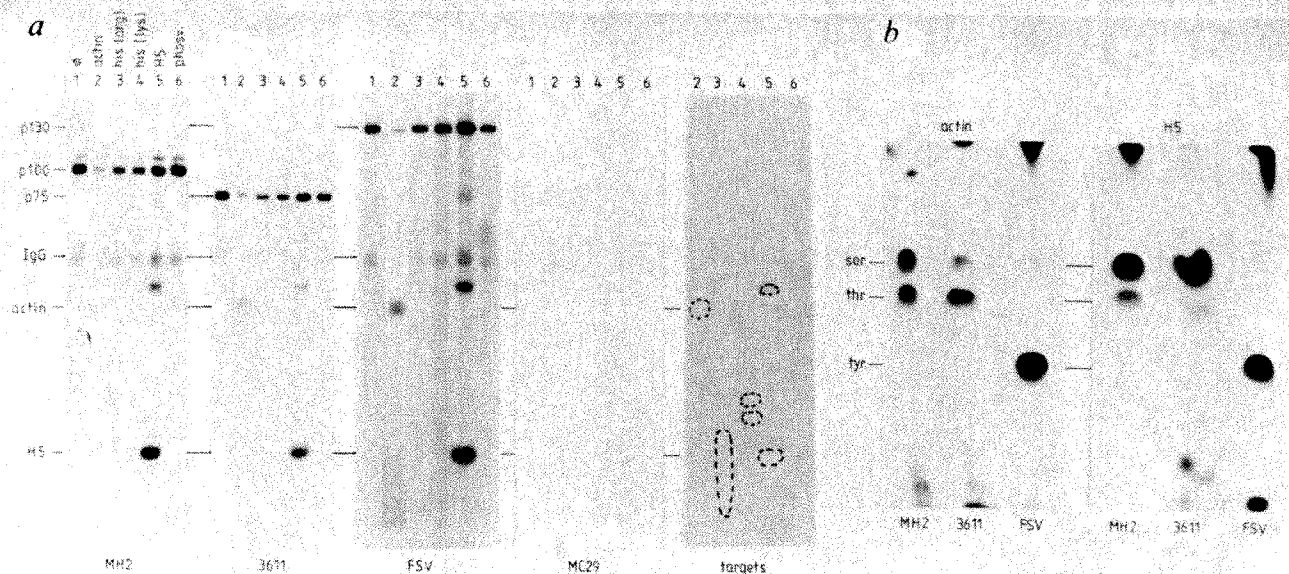


Fig. 3 Phosphorylation of substrates *in vitro*. *a*, Immunobead-immobilized enzymes were tested for their ability to phosphorylate exogenous substrates (5 μ g each, all from Sigma) *in vitro*. Protein kinase assays were performed as described in Fig. 2 legend in the presence of 10 mM $MnCl_2$. Lanes 1, no substrate; 2, actin; 3, histones, arginine-rich [his(arg)]; 4, histones, lysine-rich [his(lys)]; 5, chicken histone H5; 6, phosvitin. For control, all substrates were tested for autophosphorylation in the absence of kinases. Exposure time was as indicated using intensifier screens at $-70^\circ C$. The dotted circles indicate where the substrates migrated as determined by Coomassie blue staining. *b*, Phosphoamino acid analysis of the two substrates actin and H5 phosphorylated by MH2, 3611 and FSV protein kinases. The phosphoproteins were cut out of the gel shown in *a* and processed as described previously¹⁸. For exposure time, see Fig. 2b legend.

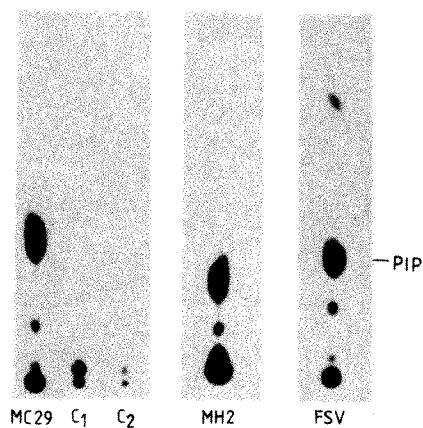


Fig. 4 TLC analyses of lipids radiolabelled with $[\gamma\text{-}^{32}P]\text{ATP}$. Immunobead-immobilized protein kinases from MH2-, FSV- and MC29-transformed cells were incubated with detergent-treated $L\text{-}\alpha$ -phosphatidylinositol 4-monophosphate. Details of the conditions have been published elsewhere¹⁴. Abbreviation: PIP, $L\text{-}\alpha$ -phosphatidylinositol; C₁, control in which lipid was omitted; C₂, control in which protein was omitted. PIP marker was co-chromatographed and traced by fluorescent light. Exposure time was 3 days at $-70^\circ C$ using intensifier screens.

phosphorylated histone H5 and actin, but phosphorylation of the other substrates was undetectable (Fig. 3a). When phosphoamino acid analysis was performed with the phosphorylated actin and H5 target molecules (Fig. 3b), p100^{gag-mil} and p75^{gag-raf} produced serine and threonine phosphorylation, while p130^{gag-fps} phosphorylated tyrosine. Some IgG also became phosphorylated. Actin seemed to inhibit autophosphorylation, while cyclic AMP inhibited all three protein kinase activities at concentrations ranging from 5 μ M to 0.5 mM (data not shown).

The recent finding that two src-related tyrosine protein kinases show lipid-phosphorylating activity^{20,21} raised the question of whether the protein kinases described here, although lacking tyrosine specificity, are also lipid kinases. Figure 4 shows that p100^{gag-mil} and p130^{gag-fps} indeed phosphorylate lipids *in vitro*. However, MC29 and normal quail cells processed by anti-p19 immunoaffinity chromatography resulted in similar levels of lipid phosphorylation under identical assay conditions (see MC29, Fig. 4).

Our results describe for the first time the presence of a serine/threonine protein kinase activity in two purified oncogene protein products. This activity is absent from quail and rat cell lines transformed by two other oncogenes, indicating that the kinase is not of cellular origin. Our results demonstrate that tyrosine phosphorylation can no longer be considered as a definitive property of oncogene-specific protein kinases. The recently observed lipid phosphorylation through the action of *src*²⁰ and *ros*²¹ oncogenes does not appear to be an intrinsic property of the protein kinases described here since similar levels of activity were also found associated with p110^{gag-myc} and normal cell controls. Phosphorylation of lipids or proteins as potential target molecules needs further evaluation. Indirect evidence suggests that the serine/threonine protein kinases described here may not be unique to *mil/raf* but may also be characteristic of the *mos* oncogene²².

We thank Drs P. Donner, T. Bunte and M. K. Owada for help in the initial part of this study. We also thank S. Richter

and S. Sukrow for technical assistance, and Dr H. Hanafusa for supplying FSV-transformed rat cell line. Dr H. Dieringer contributed stimulating discussions on the lipid assays.

Received 31 August; accepted 20 September 1984.

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Afocal apposition optics in butterfly eyes

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In most apposition compound eyes there are two components to the optical system of the ommatidium^{1,2}, the cornea and the crystalline cone. The focusing power of the cornea is well documented^{3,4} whereas the crystalline cone is usually regarded as a mere optical spacer^{5,6}; consequently, the ommatidial optics will consist of a simple focusing lens. To the contrary, we now demonstrate the existence of a complete afocal telescope in each ommatidium of butterfly apposition eyes. The optical system is an extreme variant of that found in refracting superposition eyes, thereby providing a connection between butterflies and moths.

The apposition eye consists of optically isolated units (ommatidia) each having a lens forming an inverted image (see Fig. 1 of ref. 7). In superposition eyes, the lens systems of many ommatidia cooperate to form a superimposed erect image.

Here we measured the optical properties of the ommatidia in the eye of a common Australian nymphalid butterfly, *Heteronympha merope*. The focal length, f , of the cornea (posterior nodal distance) was determined by suspending a cleaned corneal cup from a hanging drop of Ringer's solution, and measuring the size of the image of a distant object viewed through the cornea; this gave a focal length of $48.6 \pm 1.2 \mu\text{m}$ ($\pm$ s.d., $N=10$). The distance from the cornea to the image ($f' = nf$) is $67.3-70.7 \mu\text{m}$. (The refractive index used here, $n = 1.42$, is an average derived from cornea (1.52), corneal process (1.34) and cone (1.41).) In sectioned material the rhabdom tips are consistently found at a depth of $70 \mu\text{m}$, so that if the crystalline cones had no optical power, an image would be formed at the rhabdom tips. If, however, the crystalline cone acts as a lens, then the system cannot be using simple focal optics.

The crystalline cones are so small ($2.5 \mu\text{m}$ wide proximally) that most measurement techniques, including interference

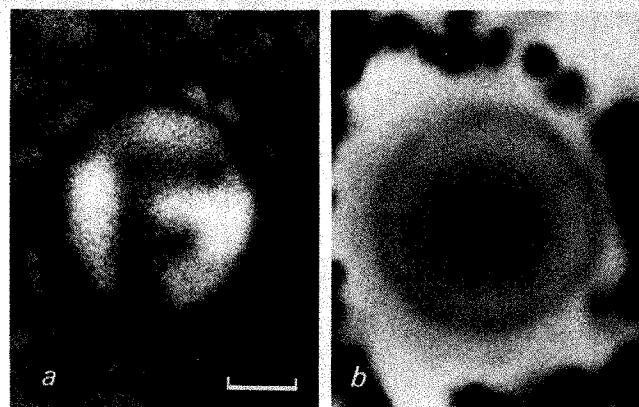


Fig. 1 Transverse sections of the proximal region of butterfly (*Heteronympha*) crystalline cones, cut in a cryotome after 1 h fixation. *a*, An image is formed of a distant letter 'F'. The inverted image is here re-inverted by the microscope. The focal length (in this case $5.1 \mu\text{m}$) was calculated from the relative size of the image. Sections with similar focal lengths were obtained from various different butterflies: *Junonia villida* (Nymphalidae), *Pieris rapae* (Pieridae), *Zizina labradus* (Lycaenidae) and *Papilio aegaeus* (Papilionidae). *b*, A protein gradient revealed by binding of toluidine blue (in *Heteronympha*). Scale bar, $1 \mu\text{m}$.

microscopy, are inadequate to resolve their optical structure. The method we finally used was as follows. Sections ($4-8 \mu\text{m}$) of stabilized material (1 h in 3% glutaraldehyde, 2% formaldehyde and 4% sucrose in 150 mM Na-cacodylate buffer) were cut in a cryotome at -14°C , then examined to determine whether they formed images (see Fig. 1*a*). Parallel-sided sections of the cones do indeed form inverted images and behave as powerful converging lenses. As judged from the unaffected pseudopupil, the optics were not damaged by the fixation. Staining of the sections with toluidine blue⁸ revealed a concentric protein gradient that is probably responsible for the focusing properties (Fig. 1*b*).

From the magnification of the images it was possible to work out the power ($1/f$) of sections taken at different levels along the cone. Figure 2*a* shows that most of the power lies in the proximal tip region; given this distribution, we were then able to trace rays through the cone by substituting each micrometre with a thin lens whose power is given by the ordinate in Fig. 2*a*. The results of this simulation are shown in Fig. 2*b, c*. Surprisingly, we found that the proximal region of the cone has exactly the right optical properties to convert the focused beam of light supplied by the cornea into a parallel beam whose diameter fits the rhabdom ($2.2 \mu\text{m}$). The weak middle region of the cone brings the converging beam to a focus $\sim 8 \mu\text{m}$ from the proximal tip of the cone, and in that $8 \mu\text{m}$ there is the equivalent of a lens powerful enough to re-collimate the focused light into a parallel beam directed down the rhabdom. It is noteworthy that this second lens is probably the most powerful known to man (0.2 megadioptries).

The optical properties of the ommatidium as a whole are accurately modelled by one spherical surface (cornea) and two thin lenses (cone) as in Fig. 3; it has the optical form of a telescope (in fact, properly known as a keplerian telescope with a Huygens eyepiece⁹). The angular magnification is 6.4, so that rays reaching the cornea at 1° to the optical axis emerge into the rhabdom at 6.4° (Fig. 2*c*). Note that the field of view of an ommatidium is now not determined by the rhabdom diameter, but by the maximum angle up to which the rhabdom will act as a light guide (in geometric optics this would be the complement of the critical angle). It also follows from the telescopic design that the rhabdom tip is imaged onto the cornea, magnified 9.1 times (the angular magnification multiplied by the refractive index inside the eye); this would mean that the $2.2 \mu\text{m}$ -wide rhabdom tip fills the central $20 \mu\text{m}$ of the $25 \mu\text{m}$ -wide facet, but in the domain of waveguide optics this cut-off will not be sharp.

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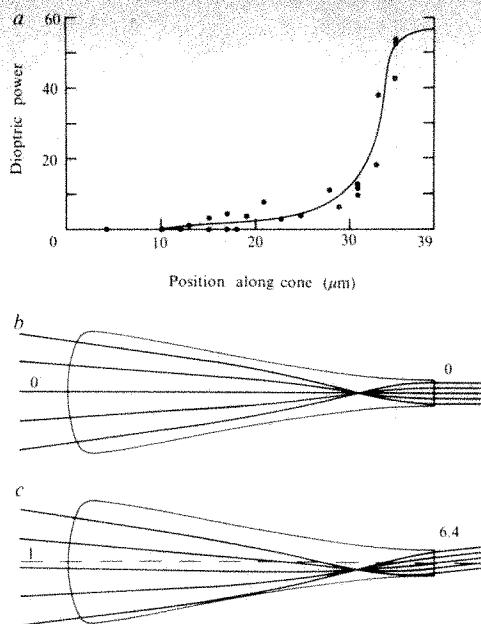


Fig. 2 Optical properties of the crystalline cone. *a*, A plot of the focusing (dioptric) power along the cone (in kilodioptries per μm). There is no power in the distal quarter but towards the proximal part the power rises monotonically, culminating as a powerful lens in the proximal tip. *b*, *c*, Rays are traced through a model cone where the focusing properties are simulated by a series of thin lenses (one per μm) with power as in *a*. The rays are parallel as they enter the cornea (not shown here) at 0° (*b*) and 1° (*c*), and emerge collimated from the cone at 0° and 6.4°, respectively.

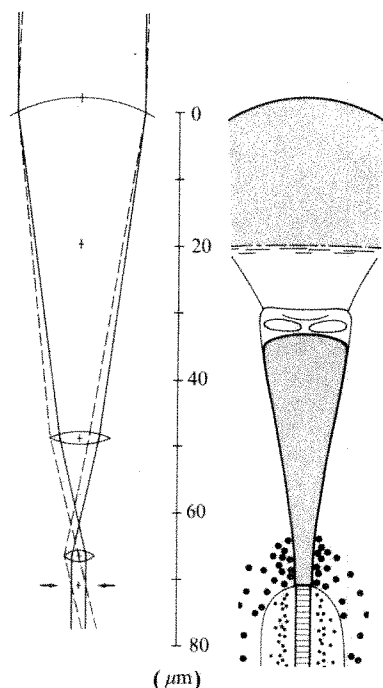


Fig. 3 The ommatidial optics is accurately modelled by one refracting surface ($f=49\text{ }\mu\text{m}$) and two thin lenses ($f=37$ and $5\text{ }\mu\text{m}$). The ray path is that of a telescope and is shown here by marginal rays entering at 0° (continuous) and 2° (dashed) to the optical axis. The rhabdom connects to the cone at the exit pupil (arrows) of the telescope. By following a pair comprising one dashed and one continuous ray, it can be seen that the rhabdom tip is imaged at the cornea. The corresponding anatomy is shown to the right: the crystalline cone is topped by its nuclei and separated from the cornea by a corneal process. The rhabdom (striped) is joined to the cone tip, and the surrounding regions are densely pigmented (represented by solid circles: large and small granules are in pigment cells and retinula cells, respectively).

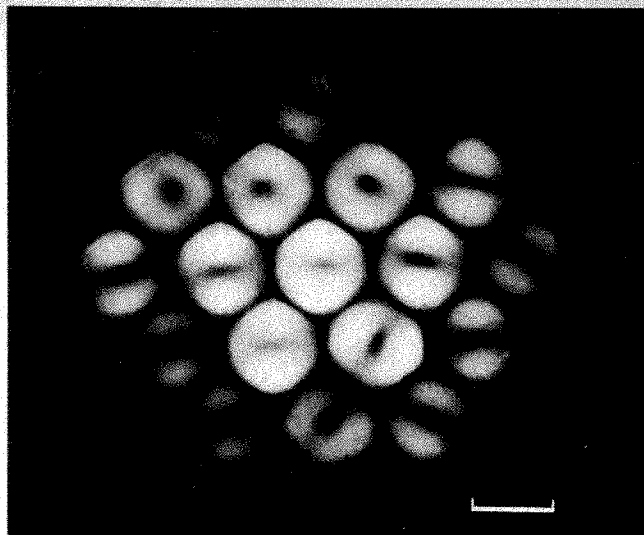


Fig. 4 Due to the reflecting tapetum, the eyes of many butterflies (here *Heteronympha*) shine brightly on incident axial illumination. The characteristic second waveguide mode, present at the rhabdom tips, is here seen imaged and magnified onto the corneal facets. Scale bar, $20\text{ }\mu\text{m}$.

A valuable check on the magnification factor is given by the waveguide nature of the rhabdom—this provides us with luminous markers in the form of the second waveguide mode (Fig. 4). Most butterflies have a reflecting tapetum at the base of each rhabdom¹⁰⁻¹², and this makes the distal rhabdom tip shine brightly on incident axial illumination. Because of the cone-lens, the pattern of waveguide modes can thus be seen magnified on the cornea. The characteristically bi-lobed or 'doughnut'-shaped second waveguide mode is only present in some butterflies, apparently those with slightly wider rhabdoms, including *Heteronympha*. The first (or fundamental) mode, which has a simple, nearly gaussian intensity profile¹³, is always present but can be extinguished selectively by viewing the eye through crossed polarizers. The number of modes that are present and their size relative to the waveguide is a function only of the waveguide parameter¹⁴

$$V = \pi d(n_1^2 - n_0^2)^{1/2} / \lambda \quad (1)$$

where n_1 and n_0 are the refractive indices inside and outside the fibre, respectively, d is the fibre diameter, and λ is the wavelength of light. The second mode is only present when $V > 2.4$ (cut-off), and its high relative intensity in *Heteronympha* indicates that V is not close to cut-off. For values of V not immediately above cut-off, the separation of the two lobes will remain fairly constant at ~ 0.65 of the waveguide diameter¹⁵. Direct measurements gave a lobe separation of 14.1 ± 0.04 ($\pm$ s.d.) μm , making the rhabdom image $21.7\text{ }\mu\text{m}$ in diameter and thus the magnification 9.9, close to the results obtained by ray-tracing. (In this calculation we have ignored diffraction effects: these will tend to blur the images of the modes somewhat, but should have little effect on the separation of the two halves of the second mode.)

Our demonstration of afocal apposition optics in butterfly ommatidia provides an interesting relation between butterfly apposition eyes and moth superposition eyes: both are afocal, but in the superposition eyes of moths and nocturnal beetles the angular magnification is low (1-2)¹⁶ and a real image is formed across a clear zone. In butterflies the angular magnification is much higher (here 6.4) and there is no clear zone to allow the formation of superposition images. It is easy to envisage a gradual transition from butterfly to moth eye or vice versa and, as recently reported for crustaceans⁷, insects now have a possible evolutionary link between apposition and superposition. However, we instead face the problem of transforming an ordinary focal apposition eye (as in bees) to an afocal one.

The final solution must await a detailed physical model comparing the relative merits of focal and afocal optics in apposition eyes.

Received 14 June; accepted 19 September 1984.

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Differential responses to male song repertoires in female songbirds implanted with oestradiol

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Considerable debate surrounds the functional and evolutionary significance of the complex song repertoires found in male passerine birds¹⁻³. A comparative study on European warblers of the genus *Acrocephalus*⁴ has suggested that sexual selection is the driving force behind the evolution of the extraordinary song complexity in this group. One species in particular, the sedge warbler *Acrocephalus schoenobaenus*, sings long, variable, continuous songs constructed from a repertoire of discrete building blocks called syllables, revealed by sonographic analysis⁵. Although a strong inverse correlation between male repertoire size and pairing date was obtained from this wild population of sedge warblers, it was difficult to control adequately for confounding variables of male or territory quality which may also influence female choice at the time of pairing. It has been demonstrated recently that female songbirds implanted with oestradiol respond to playback of recorded song in laboratory conditions⁶⁻¹⁰; the resulting displays have been used as an index of response to a variety of natural and experimental songs. Here we have used both natural recordings from an earlier field study and experimental songs to demonstrate that implanted female sedge warblers respond significantly more to playback of higher repertoire sizes in standard laboratory conditions.

The first experiment was carried out in May 1983 on eight female sedge warblers. These were taken as nestlings from the Lake Neusiedl area of Austria in July 1982 and hand-raised in the laboratory. The birds were housed in groups in appropriate photoperiod conditions for trans-Saharan migrants. In April, the photoperiod was changed suddenly to local European conditions, and in early May each female received an oestradiol implant. Next, the birds were caged separately and housed in either a soundproof chamber or an isolated quiet room for 10 days before the experiments. On the day of the experiment, each bird in its holding cage was transferred to an experimental sound proof chamber fitted with a one-way observation window and

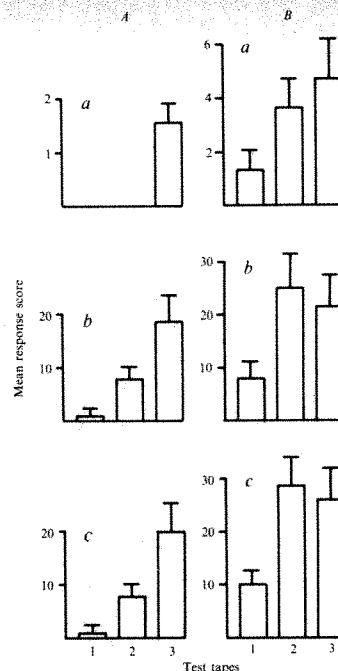


Fig. 1 Responses of eight oestradiol-implanted female sedge warblers to playback of recorded song from: **A**, three different species with different repertoire sizes; **B**, three male sedge warblers with different repertoire sizes. In **A**, test tapes consisted of 4 min of continuous song recorded from single males of: (1) blackbird *Turdus merula*, repertoire size 115; (2) reed warbler *Acrocephalus scirpaceus*, 80; (3) sedge warbler *Acrocephalus schoenobaenus*, 20. Responses measured were *a*, the number of wing vibration bouts; *b*, the number of horizontal crouches; *c*, the sum of these to give an overall response score. In **B**, the test tapes consisted of 4 min of continuous song recorded from males in an English population with repertoire sizes of: (1) low, 14; (2) medium, 24; (3) high, 41. Responses measured were the same as for **A**. The results shown are mean and standard error for the group. Each female received an implant of crystalline 17 β -oestradiol (Sigma E8E-8875) packed into Dow Corning 602-235 silastic tubing of 1.47 mm i.d. and 1.9 mm o.d. The tubing was sealed at both ends and implanted dorsally under the skin of the back where it could be seen and removed for later analysis.

a speaker. Each bird was exposed to 4 min of continuous song from males of three different species, in random order and separated by intervals of 4 min silence. The sound pressure level was standardized at 60-65 dB at a distance of 0.5 m from the speaker. Responding females became active and moved frequently into a horizontal crouching posture, occasionally vibrating their wings. Both these movements were counted separately and also totalled to give an overall response score.

Scores for the different song presentations have been compared and tested for significance using the Wilcoxon matched-pairs signed-ranks test, having first confirmed overall heterogeneity with Friedman's analysis of variance. Only sedge warbler song resulted in wing vibration, and the difference was found to be significant ($P < 0.025$) when compared with both reed warbler and blackbird (Fig. 1A). Although some crouching occurred to both reed warbler and blackbird song, the difference between these and the much larger response to sedge warbler song was highly significant ($P < 0.005$) for crouching and the total display score (Fig. 1A). The negligible baseline response to blackbird song is nevertheless an important control, as the repertoire size was nearly six times greater than that of the sedge warbler. The reed warbler had a repertoire size four times greater, and the more appreciable response obtained may in part be due to some similarities in song structure between congeneric species. Young hand-raised birds may also be expected to show rather less discrimination than wild adults, although the clear differences obtained suggest that a strong genetic preference for conspecific song structure is already established.

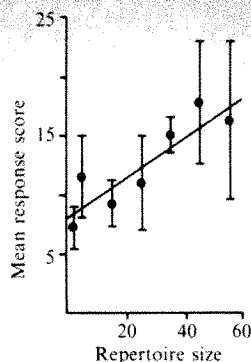


Fig. 2 The relationship between the mean response of five oestradiol-implanted female sedge warblers and the repertoire size of experimental male songs. The response score was obtained in the same way as for the first two experiments and is plotted as mean and standard error for the group. The correlation, however, is based on the original 35 data points ($r=0.342$, $P<0.05$). An independent correlation was also obtained between mean number of horizontal crouches and repertoire size ($r=0.335$, $P<0.05$). Wing vibration bouts accounted for only 10.9% of the total response, and so there were insufficient data for rigorous independent correlation analysis. However, the trend was in the same direction and, when included within the total response score, results in the slightly increased value of r . The experimental tapes were constructed from a recording of one male sedge warbler at Lake Neusiedl, Austria, by Professor A. Jilka, to whom we are most grateful. Seven tapes were made containing 4 min of continuous song from this individual, but with gradually increasing syllable repertoires of 2, 5, 15, 25, 35, 45 and 55. As the tapes were constructed from the same individual, singing rate and the total number of syllables presented remained virtually constant. The natural temporal patterning and sequencing of syllables was also maintained, and the bird merely cycled through smaller or larger repertoires of syllable types in a normal fashion.

The next experiment involved the same group of birds a few days later, and was designed to detect any preferences for conspecific males with larger repertoire sizes. Recordings of three sedge warbler males with different repertoire sizes were used to prepare 4-min tapes of continuous song as before. These were the two extremes and a middle range taken from the wild English population studied earlier. To avoid habituation to conspecific songs, the experimental design was modified so that although tapes were presented in random order only one presentation was made per day to each bird. We found that, in all three response categories, the male with the low repertoire size elicited significantly less response than the males with the medium or high repertoire ($P<0.01$ or $P<0.025$) (Fig. 1B). However, no significant differences could be found between the medium and high repertoire males. Although every attempt was made to hold constant all other variables except repertoire size, there were a number of inevitable differences between the three individuals. For example, the male with the medium repertoire sang much faster than the other two, and therefore presented the females with more syllables per unit time during the 4 min. It is likely that his faster singing rate compensated for his lower repertoire and thus abolished any differential response to the medium and high repertoire birds.

We therefore designed a final experiment in which all variables (including singing rate) were held constant, but repertoire size over a reasonable range was varied, to test whether females did in fact respond more to higher repertoires. We used one male and constructed a series of experimental tapes with gradually increasing repertoire size. The experiment was performed on a new group of five female sedge warblers taken as free-flying juveniles in the Neusiedl area in August 1983. The birds were housed and prepared as in the previous experiments and exposed to 4 min of continuous experimental song in random order on each day during May 1984. The results demonstrate a significant

correlation between repertoire size and response score (Fig. 2). We conclude that by increasing repertoire size a male sedge warbler also increases his probability of influencing female behaviour at the crucial time of female choice and pairing. These experiments support the view that sexual selection has an important role in the evolution of repertoire size and song complexity in some male birds.

Received 10 August; accepted 2 October 1984.

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Plant-inducible virulence promoter of the *Agrobacterium tumefaciens* Ti plasmid

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Agrobacterium tumefaciens is the causative agent of crown gall, a plant tumour that can arise on most species of dicotyledonous plants. The tumour-inducing capacity of the bacterium requires the presence of a large plasmid, designated the Ti plasmid^{1,2}, which itself contains two regions essential for tumour formation—the T(umour)-region and the Vir(ulence)-region³. The T-region is transferred to plant cells by an unknown mechanism, and becomes stably integrated into the plant genome⁴⁻⁹. The Vir-region has been identified by transposon mutagenesis¹⁰⁻¹⁶, but the DNA of this region has never been detected in tumour lines^{4,5}. However, *trans*-complementation of Vir mutants¹⁷ indicates that genes of the Vir-region are functional in the bacterium. Moreover, the Vir- and T-regions can be physically separated in *A. tumefaciens* without loss of tumour-inducing capacity^{18,19}. Seven loci, designated *virA-F* and *virO* (refs 17, 20-22), have been identified in the Vir-region of the octopine Ti plasmid, but their functions are unknown. As *virC* mutants in the octopine-type plasmid pTiB6 are invariably avirulent in tests on various plant species¹⁷, this gene seems to be essential for virulence and we are studying it in detail. We report here that the promoter of *virC* shows no detectable activity in *A. tumefaciens* and *Escherichia coli* K-12 grown in standard medium, but that its activity is induced by a plant product.

To investigate the promoter activity of the *virC* gene, we constructed plasmid pMP30 (Fig. 1), which contains the complete *lacZ* gene of *E. coli* coding for β -galactosidase (EC 3.2.1.23), including the translational start signals, but lacks the *lacP-lacO* regulatory sequences. *E. coli* strains harbouring this plasmid show no detectable β -galactosidase activity, indicating the absence of readthrough events from plasmid promoters. Fragments of interest can be screened for promoter activity by cloning them in the unique *Hind*III site upstream of *lacZ*.

A 2.6-kilobase (kb) *Hind*III fragment containing the promoter region and part of the structural gene of *virC* was cloned in pMP30. Figure 1 shows the orientation of this fragment in

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Fig. 1 Construction of plasmids. The broad open bar indicates the *lac* genes. The solid bars indicate DNA derived from the *Vir*-region. The *Bam*HI–*Hind*III fragment of bacteriophage Mu d (*Ap*^r, *lac*) (refs 30, 31) containing the *lac* genes Z, Y and A of *E. coli* was cloned into pK04 (ref. 32). pRAL3551 contains the *Pst*I–O fragment from the *Vir*-region¹⁷. A 2.6-kb *Hind*III fragment was isolated from this plasmid, containing parts of *virO*, the promoter of *virC* and part of the structural gene of *virC*, but not the transcriptional stop of *virC* (ref. 17). This *Hind*III fragment was cloned in pMP30, resulting in pMP31. pMP31 was transformed into *E. coli* KMBL1164 (R772) and crossed with *E. coli* KMBL1224 (*Str*^r) with a frequency of 10^{-6} . Sixteen transconjugants were crossed with *A. tumefaciens* LBA1010 Rif^r, Ti⁺ using Rif^r, Km^r, Ap^r selection in order to select for stable R772::pMP31 co-integrates. As pMP31—a pBR322 derivative—is not maintained in *A. tumefaciens*^{15,18}, Ap^r transconjugants should carry a stable co-integrate. A backcross to *E. coli* KMBL1164 confirmed this (100% co-transfer of the Ap^r and Km^r markers). One isolate, the R772::pMP31 co-integrate pMP33, was used to construct *A. tumefaciens* LBA288 (Rif^r, Ti⁺). The co-integrate R772::pMP30 (pMP32) was constructed in an analogous way. The insert shows the *Hind*III *virC* fragment present in pMP31 with the direction of transcription. Restriction endonuclease sites are indicated as follows: H, *Hind*III; B, *Bam*HI; S, *Sma*; P, *Pst*I; E, *Eco*RI; p.r. is the promoter region of *virC*; o.r.f. is the open reading frame of *virC*.

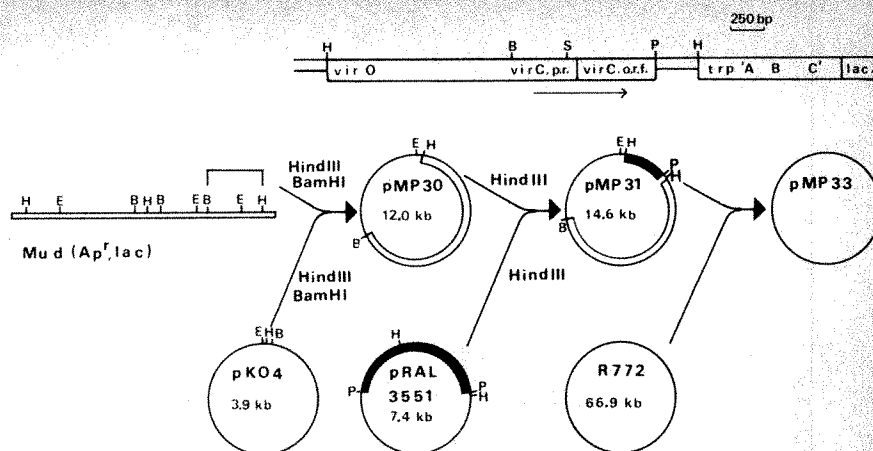


Table 1 Induction of β -galactosidase by *virC*-containing plasmids in the presence of plant exudates

Source of exudate	Bacterial strain/plasmid				No bacteria
	<i>E. coli</i> KMBL1164 pMP31	<i>E. coli</i> KMBL1164 pMP30	<i>A. tumefaciens</i> LBA1010, LBA88 pMP33	<i>A. tumefaciens</i> LBA1010, LBA88 pMP32	
<i>Pisum sativum</i> 'Finale' (Leguminosae)	++	—	+	±	—
<i>Vicia hirsuta</i> (Leguminosae)	++	—	ND	ND	—
<i>Daucus carota</i> , root culture (Umbelliferae)	++	—	+	±	—
<i>Nicotiana plumbaginifolia</i> , root culture (Solanaceae)	++	—	ND	ND	—
<i>Allium cepa</i> 'Rijnsburger' (Liliaceae)	++	—	+	±	—
<i>Zea mays</i> 'Zoete Bantammer' (Gramineae)	—	—	+ / ±	±	—
Minimal medium	—	—	±	±	—

Seeds of *P. sativum*, *V. hirsuta*, *A. cepa* and *Z. mays* were sterilized²⁷ and grown axenically for 4–8 days at 20 °C in the dark. Axenic root cultures were derived from *Agrobacterium rhizogenes* (hairy root disease)-infected plants of *N. plumbaginifolia* and from infected carrot disks (*D. carota*). Exudates were prepared by first washing the seedlings or root cultures in minimal medium for *E. coli*²⁸ or *A. tumefaciens*²⁹ for 5 min, followed by soaking for 4–5 h in the same medium. Exudates of *V. hirsuta* and *A. cepa* were prepared with 1 seedling per ml medium; exudates of *P. sativum* and *Z. mays* with 1 seedling per 3 ml medium. Exudates of root cultures were prepared with 0.1 g roots per ml medium. To eliminate plant fragments containing β -galactosidase activity, exudates were filter-sterilized before use. The pH of the medium did not change during incubation with the plant material. The sterilized exudates were inoculated with 5×10^7 bacteria ml⁻¹ and incubated for 16 h at 29 °C in the presence of 120 μ g ml⁻¹ X-gal. The bacterial concentration after incubation was $\sim 2 \times 10^8$ ml⁻¹. In a control experiment, lysates of *E. coli* (5×10^7 bacteria ml⁻¹) were tested for β -galactosidase activity with *O*-nitrophenyl- β -D-galactopyranoside (ONPG). A lysate of *E. coli* (pMP31) incubated with the exudate of *P. sativum* showed an A_{420} of 0.1–0.15 after 16 h of incubation at 37 °C with ONPG. Lysates of *E. coli* (pMP31) incubated in minimal medium and lysates of *E. coli* (pMP30) incubated in exudate showed an A_{420} of <0.001 in the same conditions. The intensity of the blue colour after reaction with X-gal was estimated and was correlated with the quantitative X-gal assay as described in Fig. 2 legend. ++, Deep blue colour ($A_{620} \geq 0.4$); +, blue colour ($A_{620} \geq 0.05$); ±, supernatant colourless ($A_{620} < 0.01$) and bacterial pellet very faint blue; —, no blue colour detectable; ND, not determined. *E. coli* KMBL1164 is F⁻ *del(lac pro)* thi; *A. tumefaciens* LBA1010 is Rif^r, Ti⁺; LBA288 is Rif^r, Ti⁺.

pMP31. It is possible that the *virO* promoter is also present in the cloned fragment, as the position of this promoter is unknown, but transcription from the *virO* promoter over the *virC* promoter into the *lacZ* gene seems unlikely. The direction of transcription of *virC*¹⁷ suggests that any β -galactosidase induction from pMP31 is due to the *virC* promoter.

Plasmid pMP31 in an *E. coli del(lac)* background showed a very weak but significant β -galactosidase activity after incubation for at least 60 h on indicator plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). No β -galactosidase activity was detected after growth for 48 h in minimal medium. pMP30, a pBR322 derivative, cannot be maintained in *A. tumefaciens*. In order to transfer and maintain pMP30 and pMP31, co-integrates with the broad host range plasmid R772 (ref. 23) were selected and crossed to *A. tumefaciens* strains LBA1010 (Rif^r, Ti⁺) and LBA288 (Rif^r, Ti⁻) (co-integrates pMP32 and pMP33, respectively). Neither co-integrate induced β -galactosidase activity in *A. tumefaciens*.

We tested whether *virC* induction was caused by the presence of plant factors (Table 1). A strong increase in *virC* promoter activity was detected in both *E. coli* and *A. tumefaciens* in the presence of exudates from a wide range of dicotyledonous varieties. The response in *E. coli* was stronger than that observed for *A. tumefaciens*, probably because the copy number of the expression vector is high (~ 40) in *E. coli* and low (~ 4) in *A. tumefaciens*. The response of the *virC* promoter on exudates of monocotyledonous plants varied with the species used. Exudates of *Allium cepa* were as effective as dicotyledonous exudates in stimulating the *virC* promoter, whereas exudates of *Zea mays* showed little stimulatory activity (Table 1). β -Galactosidase activity was undetectable in most exudates but those which did contain some activity were not used for promoter-activating studies. As the cells of the plants used contain substantial β -galactosidase activity, homogenates of plant material were not tested. No β -galactosidase activity was detected in control *E. coli* containing pMP30 or pMP32. The activity in *A.*

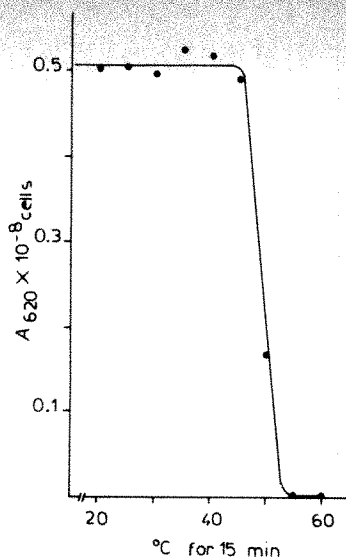


Fig. 2 Thermo-inactivation curve of *Pisum sativum* exudate. Exudate of 8-day-old seedlings was filter-sterilized and kept for 15 min at the indicated temperatures. The tubes were transferred to 29 °C and *E. coli* KMBL1164 (pMP31) and X-gal were added. After reaction, the bacteria were lysed with lysozyme, 400 $\mu\text{g ml}^{-1}$ for 10 min at 20 °C, followed by 20 mM NaOH, 0.1% SDS for 5 min at 100 °C. After centrifugation for 5 min at 15,000 r.p.m., the extinction of the coloured supernatant was determined at 620 nm. The thermo-inactivation was used in a control experiment to determine whether the induction factor works on the bacteria *per se* or on a component of the bacterial culture medium. Root exudate of *P. sativum* (filter-sterilized) and filter-sterilized culture medium of *E. coli* pMP31 were mixed 1:1 and incubated in the presence of X-gal for 3 h. No blue colour developed, indicating that bacteria are needed for β -galactosidase induction. Subsequently, the mixture was heat-inactivated for 15 min at 55 °C, cooled at 29 °C and *E. coli* pMP31 added. The bacteria were incubated for 16 h at 29 °C. No blue colour developed, indicating that the inducer works directly on the bacteria and not on a component of the bacterial medium.

tumefaciens (pMP32) was not above the very low background due to *A. tumefaciens* β -galactosidase itself. In conclusion, a factor present in plant exudates strongly activates the promoter of the *A. tumefaciens* *virC* gene.

In an attempt to identify factors that could be responsible for the promoter-activating property of exudates, several compounds which could be excreted by plants and could thus be present in exudates were tested with *E. coli* KMB1164 (pMP31). None of the compounds tested—amino acids (casamino acids 0.3%), pantothenate, nicotinic acid, *p*-aminobenzoic acid, pyridoxine, thiamine (400 ng ml^{-1} each), arabinose, lactose, galactose, sorbitol, mannitol, xylose, melibiose, cellobiose, myo-inositol (6.6 mg ml^{-1} each) and the plant growth substances kinetin (6.5 $\mu\text{g ml}^{-1}$) and naphthalene acetic acid (6.5 $\mu\text{g ml}^{-1}$)—showed promoter-inducing activity.

Control experiments (Fig. 2 legend) showed that the inducer works on the bacteria *per se* and not on a component of the bacterial culture medium. The exudate is heat-labile, showing a sharp decline in activity on incubation at 46 °C or higher and becoming completely inactivated after 15 min at 55 °C (Fig. 2). The inducing activity is not affected by treatment with RNase I, DNase, phospholipase A₂ or phospholipase D, but was abolished completely on incubation with 20 $\mu\text{g ml}^{-1}$ pronase or trypsin. Control experiments with *E. coli* CSH23-F⁺lac⁺ *del*(*lac pro*) in the presence of the *lac* inducer isopropyl- β -D-thiogalactopyranoside showed that treatment of the exudate with the enzymes did not affect the viability or β -galactosidase-inducing capacity of *E. coli*.

The exudate factor was retained on equilibrium dialysis using membranes (Spectrapore) with an indicated cut-off for globular

molecules of relative molecular mass (M_r) > 7,000. Thus, the inducer is probably proteinaceous in nature, with an estimated M_r > 7,000. Further purification and characterization of the inducer is under way in our laboratory.

Our results demonstrate for the first time the existence of a plant product that regulates the virulence of a plant pathogenic bacterium. The plant product does not require the presence of the bacterium for its appearance nor to inflict severe wounding, which is known to be a prerequisite for tumour induction. We believe that the mechanism of promoter induction is indirect because the inducer is too large to pass through the pores of the outer membrane of *E. coli*, which in general are not permeable to molecules of M_r > 700 d (for a recent review see ref. 24). The promoter described here could be of practical use in the construction of bacterial strains meant to produce substances only on contact with the plant, for example siderophores^{25,26}. The method described can be applied to the detection of other plant-regulated promoters in bacteria that interact with plants.

We thank our colleagues Drs C. A. Wijffelman, E. Pees, A. Hoekema and P. J. J. Hooykaas for stimulating discussions. Mr P. E. J. Groenewegen performed some of the experiments.

Received 21 May; accepted 20 September 1984.

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Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein?

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Calcium-dependent protease (calcium protease) is apparently involved in a variety of cellular processes^{1–3}. Here we have attempted to clarify the role and regulatory mechanism of calcium protease by analysing its structure. The complete primary structure of calcium protease (relative molecular mass (M_r) 80,000 (80K), 705 amino acids) was deduced from the nucleotide sequence of

cloned complementary DNA. The protein contains four distinct domains, and we have observed a marked similarity between the second and fourth domains and the papain-like proteases and calmodulin-like calcium-binding proteins, respectively. This finding suggests that calcium protease arose from the fusion of genes for proteins of completely different function and evolutionary origin. Further, it provides functional insight into cellular regulatory mechanisms mediated by Ca^{2+} through calcium-binding proteins.

The nucleotide sequence of calcium protease messenger RNA (Fig. 2) was deduced from the sequences of two recombinant plasmids, pCP521 (cDNA insert, 2,219 base pairs, bp) and pCP1 (cDNA insert, 2,024 bp). The two cDNAs span 3,368 bp and the nucleotide sequence in their overlapping region is identical (875 bp). DNA sequence analyses revealed total compatibility with the partial amino acid sequence of calcium (or structure of calcium) protease determined separately. Only one open frame can encode a protein of M_r 80K, and mRNA for calcium protease was detected as a single band of 3.5 kilobases (kb) by blot hybridization of poly(A) RNA from various chicken tissues (Fig. 1a). As the cDNA was 3.4 kb long, the cloned cDNA contained almost all of the mRNA sequence, but not the complete 5'-flanking sequence.

Southern analysis⁴ of total genomic DNA suggested the existence of an essentially unique gene for the calcium protease mRNA (Fig. 1b). By S_1 mapping experiments⁵, the 5' end of the exon containing the 5' region of the deduced mRNA sequence was localized at around -37 in the cloned genomic DNA sequence, allowing the 5' sequence of the mRNA to be determined. We concluded that the AUG at position 1 is the starting signal for protein synthesis (Fig. 2).

The mRNA for calcium protease encodes a protein of 705 amino acids and M_r 80,350. The N-terminus of calcium protease is blocked. N-terminal sequence analysis of the autodigestion product (M_r 78K)¹⁻³ gave a sequence identical to residues 18-26; a blocked peptide corresponding to residues 2-17 was also isolated from the digestion mixture. Thus, the N-terminus of the mature calcium protease is methionine at position 2. Carboxypeptidase Y digestion of calcium protease quantitatively liberated amino acids that were compatible with the C-terminal sequence from 692 to 705, indicating that Gly 705 is the C-terminus of the mature calcium protease. These results imply that calcium protease is not processed proteolytically.

Two types of calcium protease, one with low (m type) and one with high (μ type) affinity for Ca^{2+} , are generally found in mammalian tissues¹⁻³; chicken tissues, however, contain only m type calcium protease⁶. The enzyme comprises two components of M_r 80K and 30K¹⁻³. The 80K protein shows the same catalytic and regulatory properties as the native enzyme^{6,7}, but the role of the 30K protein remains uncertain. The 80K protein should possess at least two properties, proteolytic activity and calcium-binding activity.

Calcium protease is classified as a thiol protease⁸. Comparison of the amino acid sequence around the active site, Cys 108, with those for other thiol proteases (for example, Cys 29 in cathepsin B) revealed significant homology in a much longer sequence than previously noted (Fig. 3a)⁸. Another region around His 265 is also homologous to the sequences around the active-site His residue (His 197 in cathepsin B) of other thiol proteases. This strongly suggests that, as with other thiol proteases⁹, His 265 is essential for catalysis.

Chicken calcium protease has several calcium-binding sites, at least one of which has a low affinity for Ca^{2+} and one a high affinity (ref. 10). A typical structure, known to be a calcium-binding site, is the 'E-F hand' structure^{11,12}. All proteins characterized so far which contain high-affinity calcium-binding sites, for example, calmodulin, troponin C and myosin light chain, contain E-F hand structures, and constitute a protein superfamily with the same evolutionary origin^{13,14}. A search for such an E-F hand structure in the calcium protease sequence, using the method of Tufty and Kretsinger¹², revealed four candidates, at positions 589, 619, 654 and 684. Of these, those structures

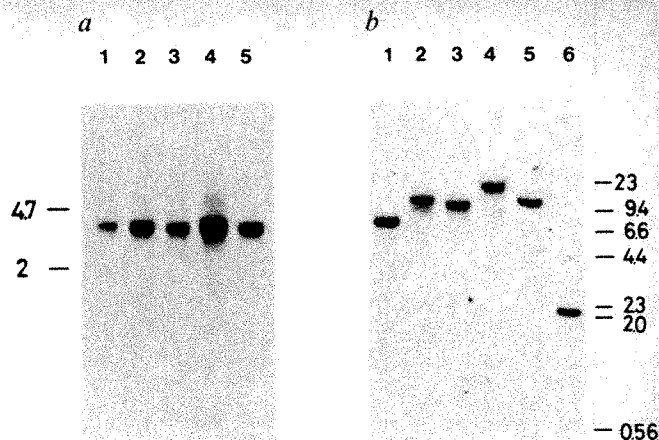


Fig. 1 a, Identification of calcium protease mRNA in various chicken tissues by blot hybridization using a cDNA for chicken calcium protease as a probe. Lanes: 1, brain; 2, spleen; 3, muscle; 4, lung; 5, kidney. The size marker was mouse ribosomal RNA. b, Identification of the calcium protease gene by blot hybridization analysis of chicken cellular DNA. Lanes: 1, *Eco*RI and *Hind*III; 2, *Hind*III; 3, *Hind*III and *Bam*HI; 4, *Bam*HI; 5, *Bam*HI and *Eco*RI; 6, *Pst*I. Size markers were λ *Hind*III digests. Numbers on the right and left-hand sides are size markers (kb).

Methods: a, Poly(A) RNA (5 μ g) was denatured with 2.2 M formaldehyde and 50% formamide, electrophoresed on 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose paper²². The hybridization probe used was a *Pst*I fragment (residues 31-1,128). b, Total chicken cellular DNA was digested with restriction enzymes, electrophoresed on a 0.8% agarose gel and subjected to blot hybridization analysis⁴ using a *Pst*I fragment (residues 31-1,128) as a probe.

starting at residues 589, 619 and 684 contain highly conserved sequences (33-50%) seen in 30 established calcium-binding sites, and consisting of 12 amino acids¹⁵ (typical examples are given in Fig. 3b). Their predicted secondary structure suggests that these potential calcium-binding sequences are located between two α -helical regions. These sites should therefore function as calcium-binding sites in calcium protease. All are found at the C-terminal region of calcium protease, and their spacing along the protease chain resembles that in other calcium-binding proteins (Figs 3b, 4).

We can now develop a schematic structure for calcium protease which is composed of four distinct domains: domain I, residues 1-80; domain II, residues 81-320; domain III, residues 321-560, and domain IV, residues 561-705 (Fig. 4). The function of domains II and IV was clarified using sequence homology and previously reported results, but the function of domains I and III remains unknown; perhaps they act as intramolecular regulators of the catalytic activity residing in domain II or as binding sites for the 30K protein, inhibitor protein¹⁻³ or activator protein¹⁻³.

Calcium is essential for the proteolytic activity of calcium protease; thus, the activity of domain II is suppressed—possibly by domains I and/or III—when it is incorporated in the complete calcium protease molecule. The role of the calcium-binding domain (domain IV) is to derepress the protease activity of domain II. In this respect, the role of domain IV is similar to that of calmodulin which, in the presence Ca^{2+} , activates (or derepresses) enzymes such as phosphodiesterase¹⁷ or phosphorylase b kinase¹⁸ by means of protein-protein interaction. However, there is an important difference between the regulation of enzyme activity by the calcium-binding domain of calcium protease and by calmodulin. This is that the calcium protease calcium-binding domain (domain IV) is covalently linked to the target protease portion (domain II) through domain III. This

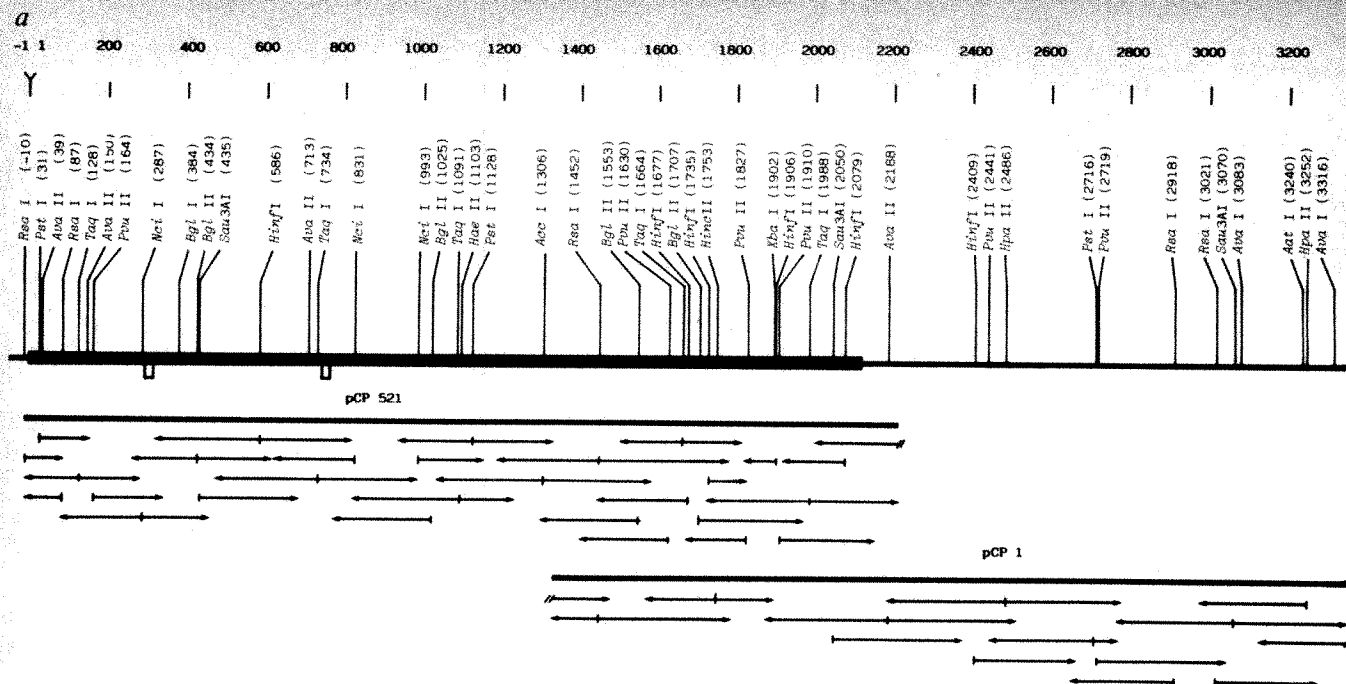
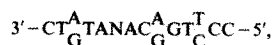


Fig. 2 a, Restriction map and sequencing strategy for the calcium protease cDNA inserts in clones pCP521 and pCP1. The restriction map shows only relevant sites. Nucleotide residues are numbered in the 5' to 3' direction, beginning with the first residue of the AUG triplet encoding the initiation methionine, and the residues on the 5' side of residue 1 are indicated by negative numbers. The poly(dA)·poly(dT) tract and poly(dG)·poly(dC) tail are not included in the map. The sequence of the protein-coding region is indicated by a solid box, and those corresponding to the chemically synthesized hybridization probes are indicated by open boxes. DNA sequencing was carried out by the procedure of Maxam and Gilbert²⁰. The direction and extent of sequence determinations are shown by horizontal arrows under each clone used; the sites of 5'-end labelling are indicated by short vertical lines at the end of arrows. The slash marks at the ends of arrows mean that the site of 5'-end labelling was the *Pst*I site at the end of the poly(dG)·poly(dC) tail. **b**, Nucleotide sequence of chicken skeletal muscle calcium protease mRNA and the deduced amino acid sequence. The sequence upstream of -11 was deduced from that of cloned genomic DNA. The 5'-terminal sequence presented does not extend to the 5' end of the mRNA (see text), whereas the 3'-terminal sequence is followed by a poly(A) tract of 16 nucleotides connected to the GC tail, thus representing the complete sequence of this region. The sequence, UAG, at -21 indicates the termination codon preceding the initiation codon at 1 in phase. The AAUAAA sequence that usually precedes the poly(A) sequence starts at residue 3,324. The predicted amino acid sequence is shown below the nucleotide sequence, and amino acid residues are numbered beginning with the initiation methionine. Amino acid sequences of various fragments of calcium protease determined by micro-scale Edman degradation are shown by small horizontal arrows. Amino acid composition of the N-terminal peptide liberated from calcium protease by autodigestion and amino acids liberated from the C-terminus by digestion with carboxypeptidase Y are underlined.

Methods: **a**, Two sets of synthetic oligodeoxyribonucleotides were used as hybridization probes. One (no. 20) was a mixture of 16 tetradecamers,



synthesized and used as two pools containing T or C at the ninth position, designated as N, which correspond to the amino acid sequence at positions 252-256 (b). The other (no. 30) was a mixture of 24 tetradecamers,



synthesized and used as two pools containing A/T or G at the sixth position, designated as N, which correspond to the amino acid sequence at positions 99-103 (see b). Total RNA was extracted from chicken skeletal muscle²¹, and poly(A) RNA was isolated by oligo(dT)-cellulose chromatography²². Poly(A) RNA was fractionated by sucrose density centrifugation and assayed by translation in a rabbit reticulocyte lysate system²². Translation products were analysed by SDS-gel electrophoresis after immunoprecipitation with rabbit antibody against chicken skeletal muscle calcium protease. Fractions giving an 80K protein band were pooled and used as a template for cDNA synthesis. Double-stranded cDNA was prepared by standard methods using poly(A) RNA from the 25-26S fraction²². cDNA >1.5 kb was isolated by agarose gel electrophoresis and annealed with plasmid pBR322 by the GC-tailing procedure²². *Escherichia coli* χ 1776 was used for transformation, and tetracycline-resistant transformants were screened *in situ* by hybridization²² at 40 °C or at 37 °C with the synthetic oligodeoxyribonucleotide mixtures no. 20 or no. 30, which were labelled with ³²P at the 5' end²⁰, respectively. One recombinant clone (pCP521) which hybridized with both of the two probes was isolated from 1 × 10⁶ transformants. Another clone (pCP1) was isolated from the same cDNA library by hybridization with a mixture of two *Pst*I fragments (31-1,128 and 1,128-end of the GC stretch of the 3' end) of the pCP521 insert which was labelled by nick-translation. Three hybridization-positive transformants were isolated; one of them (pCP1) was subjected to DNA sequencing in addition to pCP521. Both DNA strands were sequenced and all the restriction sites used for sequencing were read through by sequencing begun at other restriction sites. **b**, A chicken genomic DNA clone, λ CP115, was isolated from the chicken genomic DNA library provided by Dr J. D. Engel. To determine the 5'-flanking sequence of calcium protease mRNA, which was not cloned into the cDNA recombinant pCP521, the genomic DNA was analysed by sequencing and S₁ nuclease mapping⁵. A single-stranded DNA probe spanning from -354 (*Eco*RI site) to 39 (*Ava*II site) 5'-end-labelled at the *Ava*II site was hybridized with poly(A) RNA from chicken skeletal muscle and the S₁-resistant DNA was analysed.

regulation is more simple and the enzyme activity more directly controlled by Ca²⁺ than in the case of calmodulin, where it exists dissociated from the target protein or as a subunit of the target enzyme (as, for example, in phosphorylase *b* kinase¹⁸).

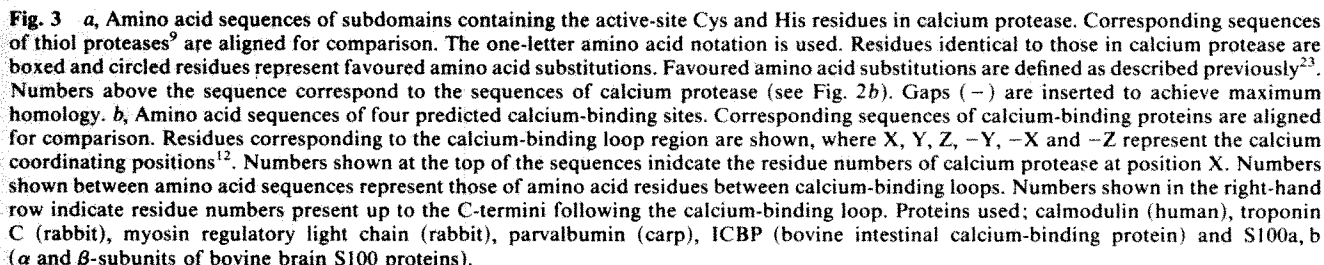
Calcium protease contains two domains that are significantly homologous to thiol proteases and calcium-binding proteins. Although the homology of each domain with its counterpart does not exceed 20-25%, the spacing of functional subdomains clearly indicates their structural homology. This implies that calcium protease is a member of two distinct protein super-families.

There are many examples of the fusion of duplicated genes

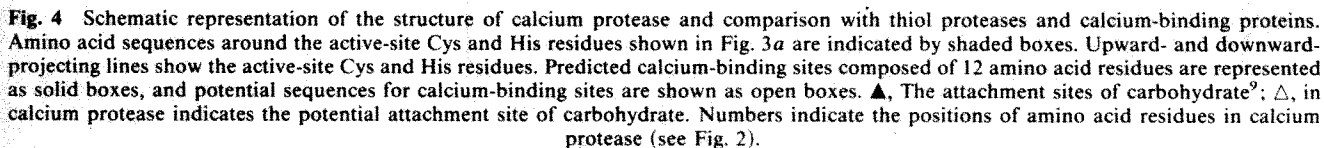
during protein evolution¹⁹. However, there have been few reports of gene fusion of proteins with different functions and evolutionary origins, probably because the structure of proteins that have multiple distinct functions remains poorly understood. The results presented here suggest strongly that calcium protease arose by fusion of genes for thiol proteases and calcium-binding proteins, although the possibility that this protease arose by convergent evolution cannot be excluded. Comparison of the gene structure of these protein families should elucidate the evolution of calcium protease.

We thank Professors K. Imahori and S. Ebashi for their encouragement and advice, and Dr J. D. Engel for providing

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 Met Met Pro Phe Gly
 80 100 120
 AAC AAC GCU UAG AAC UAC CUC AAC CAG GAC UAU GAA GCC CUC AAG AAC
 Asp Asp Ala Val Lys Tyr Leu Asn Gln Asp Tyr Glu Ala Leu Lys Gly Thr
 30 40
 180 200 220
 GCC CUC GGC UUC AAG GAG CUG GGG CCA UAC UCC AGC AAG ACA CGG GGC
 Ala Leu Gly Phe Lys Glu Leu Gly Pro Tyr Ser Ser Lys Thr Arg Gly
 60 70
 300 320
 GCA ACC CGG ACA GAU AUC UGC CAA GGA GCU CUG GGU GAC UGC UGG CUG
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 400 420
 CAG GGG GAG AGC UUC CAG GAG GAC UAC GCU GGC AUC UUC CAC UUC CAG
 His Gly Gln Ser Phe Gln Glu Asp Tyr Ala Gly Ile Phe His Phe Glu
 130 140
 500 520
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 600 620 640
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 200 210
 720 740
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 820 840
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 Thr Ala Phe Lys Asp Val Asn Tyr Arg Gly Gln Glu Gln Leu Ile
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 Ser Glu Trp Asp Asn Ile Asp Pro Ser Asp Arg Glu Glu Leu Gln Leu
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 PHE LEU VAL ALA LEU MET
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 Gly Ser Gln Asn Val His Leu Lys Lys Asp Phe Phe Leu Arg Asn Gly
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 6160 6180 62



Methods: b, E-F hand calcium-binding sites were searched for by the method of Tufty and Kretsinger¹² in the calcium protease sequence. The alignment for the test sequence, that is, the number of residues identical to the test sequence at 16 positions within and around the calcium-binding loops^{12,24}, was 11 for each of the four sequences. The second class of high-score sequences lie at positions 154, 396 and 545, with scores of 9. Scores of established calcium-binding sequences vary from 12 (domain III of carp and rabbit parvalbumin) to 16 (domain II of rabbit troponin C). The scores of potential calcium-binding sequences in rabbit myosin regulatory light chain (II, III and IV) were 10, 10 and 11, respectively.



Received 20 July; accepted 21 September 1984.

- the chicken genomic DNA library. This work was supported in part by research grants from the Ministry of Education, Science and Culture, and by a grant-in-aid of new drug development from the Ministry of Health and Welfare, Japan.
- Received 20 July; accepted 21 September 1984.
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Holland's Earth

James E. Lovelock

The Chemical Evolution of the Atmosphere and Oceans.

By Heinrich D. Holland.

Princeton University Press: 1984. Pp.582. Hbk \$97.50, £80.70; pbk \$24.50, £20.30.

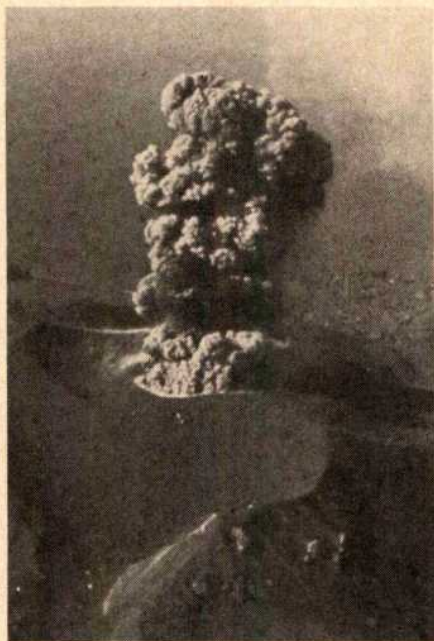
THERE is an almost sensual pleasure to be had from collecting small rare objects, such as stamps, books or rocks. Some modern books on natural history can delight the reader in the same way, but it is unusual for a textbook to elicit this feeling. *The Chemical Evolution of the Atmosphere and Oceans* is an exception.

Professor Holland's view of the evolution of the Earth — where the constancy of climate and composition over geological time is a straightforward consequence of the working of chemical and physical forces — differs sharply from mine that sees in the constancy a homeostasis maintained for and by the biota. That this difference is mutual is noted courteously but firmly in his closing pages. In no way does this difference of interpretation alter my pleasure from reading the book, nor my admiration of its authoritative excellence — science can benefit from the personal dialectical approach and guesses are needed to start the iterations that may converge towards the unattainable goal of truth. The book is enhanced by clear and comprehensible graphs and tables, and at times reads more like the report of a research project than a textbook.

It begins with four chapters about the origins of the Earth and on what is known of the Hadean. It is easy to forget that this turbulent period occupied nearly one-quarter of the Earth's history, whereas human beings have occupied only the past thousandth of it. Models of the Hadean are thin and rather speculative but here are displayed the few solid facts that are available. It is a source of wonder that one of the most certain of these is the earliest; namely, that much of the material of the Earth came from a supernova explosion 4.55 Gyr ago. The evidence from the distribution of radioactive and stable isotopes, the fall-out of that vast initial nuclear explosion, is used extensively throughout the book. Just as loud and angry voices carry further than quiet conversation, there is a tendency to concentrate on events that are violent and stormy rather than on the calmer periods in between; yet it could have been during a calm spell at the end of the Hadean that life assembled itself. Essential for theories of the origin of life is information on the structure and composition of the prebiotic atmosphere and oceans. It seems that the new orthodoxy is still with us and that the atmosphere at the end of the Hadean was dominated by CO₂ and neutral to slightly reducing. The oceans by contrast were buffered at a low

redox potential by an abundance of soluble electron-rich species of elements such as iron and sulphur.

As the book moves on into the Archean, the emergence of life is noted but curiously kept at low key as if the author considered it to be impolite or improper to discuss biological matters too deeply in a book on chemical evolution. Perhaps, though, the reluctance to become involved with the fashionable science of biogeochemistry is wise. There have been numerous attempts



Atmospheric input — Krakatoa rumbles on, one hundred years after the original eruption.

to put the biota in a box and assign to it inputs and outputs, an operation analogous to that of making a materials and energy balance of a living animal. Something may be learnt from these exercises but they can offer nothing to explain the potential of living systems for homeostasis nor can they predict the course of their evolution. An interdisciplinary approach to earth science is more in need of contributions from geophysiology than from biogeochemistry.

This beloved data-bank of a book that never considers things beyond the limits of decent speculation also never ceases to present its data in a way that tempts speculation far beyond those limits. To nowhere does this apply more than the chapter on terrestrial environments. The environmental factors that determined the speciation of uranium at the end of the Archean are discussed at length yet there is

no word about the strange affair of the natural nuclear reactors at what is now Oklo in Africa. There, 2.35 Gyr ago, uranium was sufficiently concentrated and purified to exceed the critical mass needed for a working nuclear reactor. Did the primitive artifice of dumb prokaryotic miners equal our proud science and technology? And what does the existence of that reactor then tell us of the atmosphere of those times?

The personal character of the book is strongest in the final chapter that deals with the Phanerozoic. The fascinating question of why the composition of the sea has stayed so constant is made significant by the skilful marshalling of the evidence. All is included, even the poetic names of those extraordinary crystalline rocks of the evaporites; such as polyhalite that bears no halogens or sylvite that does. From the constancy of the composition of sea water we move on to the constancy of the abundance of oxygen in the recent atmosphere. I could not help wondering here why the evidence of the presence of charcoal and of fossilized wood in sediments is not mentioned as setting quite sharp limits on the possible range of oxygen. If charcoal is a fossil indicator of fires, the fires can only have occurred when oxygen was within the range 15% to 25%.

The evolution of the Earth as a planet is vastly different from that of the other terrestrial planets. As Anderson has recently observed (*Science* 223, 347–355; 1984), plate tectonics are unique to the Earth and made possible only by the presence of limestone, which in turn is almost wholly a product of the biota. The geological community is only now beginning to take on the difficult problem of explaining the effects of the presence of the biota on the evolution of the Earth, and it will be some time before the biological presence is considered as a driver rather than a passenger. There are still formidable barriers to interdisciplinary thinking.

As a young scientist I was advised to read original papers rather than textbooks. Times have changed — nowadays too many published papers are either otiose or just plain wrong. How can those who seek information outside their speciality sift the good from the bad? This is where a book such as this is invaluable to a general scientific readership. It is well and clearly written, providing an authoritative summation of information about the Earth from the viewpoint of a geologist and a chemist, and also serves as a guide to the key original sources. But most of all it contains a constant supply of robust but finely tested facts and decent speculations that can be used for building images and models. It will be my companion for many years to come. □

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Building the modern main-frame

John Hendry

The Computer Comes of Age: The People, the Hardware, and the Software.

By R. Moreau. Translated by Jack Howlett.

MIT Press: 1984. Pp.227.

\$19.95, £20.85.

THERE are remarkably few books on the recent history of computing. Accounts of the prehistory, from Babbage to the Second World War, abound, as do reminiscences of the pioneering wartime and immediate post-war projects. A start has even been made — for example by Nancy Stern in *From ENIAC to UNIVAC* — on a genuinely historical analysis of these projects. But there is still precious little on events since 1950, and virtually nothing in accessible book form.

The Computer Comes of Age, a translation of René Moreau's 1981 book *Ainsi Naquit l'Informatique* (French birth corresponding, apparently, to English adulthood!), is the first significant exception to this rule. The work is a chronicle rather than a history and the style of presentation, though an improvement on the French edition, does not make for easy reading. The report-like format, with short headed sections, many only a paragraph long, makes few concessions to narrative continuity. Indeed, as one advances through the book and the technological developments, all of which are explained for the benefit of the non-specialist, get more complex, one becomes increasingly aware of a similarity with computer training manuals. The focus is almost wholly on the technology, with nothing on the social or economic aspects of computer development, and because the work is based largely on "common knowledge" rather than historical research it is far from being authoritative. Finally, even Moreau closes his account with the arrival of the IBM series 360 computers in 1963, a recent event by normal historical standards, but a very distant one if one considers how much has happened since that time.

In short, then, this is not really a book for casual reading, for those interested more in the computer age than in the computers (and computing languages) themselves, or for those seeking a definitive history. Nonetheless, within its very reasonable limits it is a valuable work.

The strength of the book lies in its comprehensive coverage of the myriad of technical developments leading up to what was, in effect, the modern main-frame computer. From ferrite-core memories to front-end processors and from non-local concurrency to the characteristics of COBOL, pretty well every important

development of the period is covered, be it in hardware, software, peripherals or operating methods. Almost all the most interesting computers of the period are described, albeit briefly, with mention of their particular innovations and the degrees to which they incorporated emergent technologies. The often-neglected problems of operation and communication, and the development of programming languages, are also brought in.

It is, however, a pity that the laudable effort to make the subject accessible could

not have been accompanied by a similar effort to make it attractive; as it stands, the book is unlikely to appeal greatly to the computer-non-literate population for whom it was apparently intended. But the fact is that no one has yet done any better, or even anywhere near as well in tackling the subject. Until they do, René Moreau's book well deserves whatever success it may find. □

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Principle patterns

Brian Goodwin

Pattern Formation: A Primer in Developmental Biology.

Edited by George M. Malacinski and Susan V. Bryant.

Macmillan, New York/Collier-Macmillan, London: 1984. Pp.626. \$58, £55.

GEORGE Malacinski and Susan Bryant have brought together an excellent collection of essays on pattern formation. No volume could be comprehensive and definitive in such a diverse and rapidly-moving field, but this one comes close. Not only is the range of organisms treated very extensive, from the ciliate protozoa and *Volvox* to higher plants and animals (though nothing on mammals!); but the conceptual scope is also broad, including work informed largely by classical embryological concepts, sophisticated analytical treatments by the model-builders, and detailed analyses of the cellular, genetic and molecular mechanisms involved in pattern formation. Few subjects can boast such an apposite pluralism, for the whole organism together with all its parts continue to exist as real structures in developmental biology. Dominant themes nevertheless come and go. *Pattern Formation* celebrates a period of research whose intellectual liveliness, balance between empirical discovery and conceptual interpretation, and sheer enjoyability owe much to the personality of one individual, Lewis Wolpert. It is a further tribute that this volume has within it not only material that derives directly from his work, but indications of what type of analytical framework may supersede it.

The book starts with summaries of discussions between Malacinski and some of the contributors, the first of whom states that Wolpert's theory of positional information is not a falsifiable theory, but a concept, a framework for the interpretation of phenomena. In his article with Stein, however, Wolpert says that "the concept of positional information was explicitly formulated by Driesch", arising from his observation that sea urchin blastomeres are

totipotent, their actual fates therefore depending upon their spatial relations to other cells in the embryo. This was a testable theory, and my understanding is that Wolpert takes the same view of his particular formulation of Driesch's proposition. Certainly, several contributors treat it as such and test it critically against empirical evidence. It is worth noting in passing that in converting Driesch's concept of relational spatial order into the notion of a coordinate system within the organism, Wolpert constructed a conceptual framework, via the notion of free and unconstrained interpretation of "positional values" by species-specific genetic programmes, which opened the door wide to the Darwinian principle that any organismic form is possible, natural selection then making its choice from the continuum. Driesch vehemently opposed this view, planting his banner firmly with the rational morphologists and "laws of form".

Accounts of the search for generative rules or laws operating during development occupy a significant part of this volume, as Susan Bryant observes in her introductory comments. This reflects an important transition in the evolution of positional information models from their early, virtually unconstrained form to ones embodying abstract generative rules such as those of the polar coordinate model (well represented in articles by Held and Bryant on *Drosophila*, and by French, Holder and Javois on insect, amphibian and chick limbs respectively). Perhaps the most significant consequence of this model is its demonstration that simple rules with empirical consequences can be formulated without reference to molecules, or even cells, capturing principles of spatial organization that extend across an impressive range of taxa. The ingenious application by Frankel of these principles to the explanation of supernumerary oral apparatuses with reversed symmetry in *Tetrahymena* reminds us that pattern formation is not, in fact, dependent upon cellular interactions. Thus we seem to catch a glimpse of universal principles, the general spatial properties of which are developed with characteristic incision by Winfree.

The richness of morphogenesis, analytically explored in "simple" forms such as *Volvox* (Sumper), *Dictyostelium*

(MacWilliams), *Hydra* (Bode and Bode), and in more complex organisms such as insects (Sander; Anderson and Nüsslein-Volhard), higher plants (Sachs, McDaniel), sharks (Reif), amphibians (Malacinski, Slack, Cooke, Stocum and Fallon) and chicks (Dhouailly), force an elaboration of concepts beyond either the original positional information theory or the polar coordinate model. Here we find a profusion of developmental mechanisms, some classical and some modern, all very clearly elaborated. Both Kauffman and Meinhardt achieve some success in accounting for certain aspects of these phenomena by the discovery of new properties of old models, and by adding dynamical features with switching logic and recursive properties to generate structures such as segments and compartments, and models of distal transformation in insects.

But for me the high point of the volume, whence one catches a glimpse of a way of putting together all that has come before but within a somewhat different scheme, arose from a certain reading of the chapters by Karlsson, Poethig and Nuccitelli — the first on *Drosophila*, the second on angiosperm leaf morphogenesis and the third on electrical fields in morphogenesis (plant and animal, unicellular and multicellular). The first two authors interpret pattern formation in terms of fields of mechanical force (mediated by the cytoskeleton) that both guide and are guided by patterns of cell division and differentiation in *Drosophila* imaginal discs and in leaf meristems; while the Nuccitelli paper on ionic currents indicates the dynamic boundary conditions for affecting the state of the cytoskeleton and cell metabolism.

Neither Karlsson nor Poethig sees evidence in their material for either a logical gene-regulated decision process resulting in spatial patterns, or for gradients or "coordinate-systems" that spatially control such decisions. Since Sydney Brenner, somewhat impishly, has recently said about the explanation of pattern formation by molecular mechanisms of gene control: "I doubt whether anyone believes that anymore", we can now feel free to seek alternatives. One possibility is to understand morphogenesis as a unitary process in which global mechanical fields (of strain, or strain-rate, for example), acting through and on both the cytoskeleton and cell membranes, result in ion fluxes and associated elastic and plastic deformations of tissue that themselves generate further stresses, ion flows and other biochemical changes (including, of course, gene activities), with reciprocal interactions. After an extended exploration of the information and programming metaphors, the subject may be returning to the themes of *Entwicklungsmechanik*; which would have pleased Driesch. □

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Trains of thought

John C. Marshall

Vehicles: Experiments in Synthetic Psychology.

By Valentino Braitenberg.

MIT Press: 1984. Pp.152.

\$14.95, £15.60.

WILLIAM Thomson, Lord Kelvin, is reputed to have remarked: "If you can make a mechanical model of it you understand it, and if you can't you don't". Kelvin was, of course, talking about the role of models in physics (and thinking about the success of his tidal wave predictor), but he could equally well have had psychological models in mind.

There is a tradition in theoretical psychology, extending back some two millennia, whereby the explanation of behaviour is explicitly equated with the construction of "toys" that exhibit the behaviour in question. Some of the outstanding contributors to this enterprise include Heron of Alexandria, who built a complex automaton wherein, on the lifting of a golden apple, a miniature Hercules shot a snake that then hissed; Albertus Magnus, whose *magnum opus*, a life-sized automatic servant, was destroyed, so legend goes, by Thomas Aquinas who suspected it to be the Devil's work; Jacques de Vaucanson, the constructor of an eating, drinking and quacking artificial duck, and of a flute-player that out-performed many human virtuosi; and the Jaquet-Droz (*père et fils*), whose dolls could write and draw with amazing facility. Like Magnus, the elder Droz and his models suffered a fate more extreme than failure to obtain tenure. Following a public exhibition of the writing-boy, Hermann von Helmholtz reminds us that "... this boy and its maker, being suspected of the black art, lay for a time in the Spanish Inquisition, and with difficulty obtained their freedom".

Closer to our own time, Grey Walter and Claude Shannon devised, respectively, tortoises and mice that ran mazes in electronic instantiation of the laws of classical learning-theory. To this distinguished line of artificial intelligentsia we must now add Valentino Braitenberg, a director of the Max Planck Institute of Biological Cybernetics at Tübingen.

Professor Braitenberg's *Vehicles* is precisely that; a series of 14 little vehicles, with surprisingly life-like properties. The sequence begins with a simple bug where one sensor is connected to one motor such that "the more there is of the quality to which the sensor is tuned, the faster the motor goes" and ends with creatures of more Rococo wiring that appear to demonstrate foresight, egotism and optimism. Each successive creature embodies the critical structures of all previous models and adds one further feature; the vehicles thereby illustrate a single line of evolu-

tionary ascent and painlessly introduce the unsuspecting reader to the basic concepts of self-regulating machines. Throughout, Professor Braitenberg writes in a dry, tongue-in-cheek fashion, poking sly fun at any philosopher (or Reith lecturer) whose hand is too unsteady to wield Occam's razor. He is thus a worthy successor to Julien Offray de la Mettrie, who in his *L'Homme Machine* of 1748, argued that

... since all the faculties of the soul depend to such a degree on the proper organization of the brain and of the whole body, that apparently they are but this organization itself, the soul is clearly an enlightened machine.

For both la Mettrie and Braitenberg, it's Chinese rooms all the way down.

In the main text, Professor Braitenberg's humour serves to illuminate his serious moral, "the law of uphill analysis and downhill invention", that is:

It is pleasurable and easy to create little machines that do certain tricks. It is also quite easy to observe the full repertoire of behavior of these machines — even if it goes beyond what we had originally planned, as it often does. But it is much more difficult to start from the outside and to try to guess internal structure just from the observation of behavior.

Worse,

it is actually impossible in theory to determine exactly what the hidden mechanism is without opening the box, since there are always many different mechanisms with identical behavior.

Accordingly, Professor Braitenberg provides an extensive appendix of biological notes on his vehicles. Here he sketches the analogies between his creatures and those of the given, natural world, leading one lightly into the complexities of current neuroanatomy and neurophysiology. The transition between the stark formalism of the hardware vehicles and the cell-synapse-circuit wetware that they mimic is mediated by a portfolio of phantasmagoric drawings by Maciek Albrecht.

For both amusement and enlightenment, *Vehicles* can be unhesitatingly recommended to anyone (student or teacher) involved in courses on "Brain and Behaviour". The volume would also make a marvellous Christmas present for any intelligent teenager interested in either computing or animal-watching, or better, both. □

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New in paperback

● *The Structure of Time* by W. H. Newton-Smith. Publisher is Routledge & Kegan Paul, price is £7.95, \$14.95.

● *Curious Naturalists* by Niko Tinbergen, a reprint of the revised (1974) edition of the book. Publisher is The University of Massachusetts Press, price is \$9.95.

● *Plant Biotechnology*, edited by S. H. Mantell and H. Smith, Vol. 18 in the seminar series of the Society for Experimental Biology. Publisher is Cambridge University Press, price is £12.50, \$19.95.

Sense of rays

Richard L. Garwin

Beam Weapons: The Next Arms Race.

By Jeff Hecht.

Plenum: 1984. Pp.363. \$17.95, £17.10.

IN FEW areas of science and politics has public interest grown as rapidly as in that of directed energy weapons, especially as applied to space defence and anti-satellite attack. Key to this surge of awareness is President Reagan's speech of 23 March 1983, in which he called for a research programme to "render nuclear weapons impotent and obsolete" by the ability to "intercept and destroy strategic ballistic missiles before they reached our own soil or that of our allies". Jeff Hecht's book was at the publisher's in draft form on that date, but the President's "Strategic Defense Initiative" (SDI) (or "Star Wars") speech figures prominently in the updated text.

With a degree in electronic engineering from California Institute of Technology, and seven years as managing editor of *Laser Focus*, Hecht is well placed to interpret for a popular audience the development and potential of beam weapons — that is, laser beams of infrared, visible or X-radiation; charged or neutral particle beams of protons, electrons or hydrogen atoms; and beams of microwave radiation. He makes a valiant attempt to report myth as myth and fact as fact. Unfortunately the jobs of popularizer and decision-maker alike are seriously impeded by an unusual lack of substantive unclassified material from the United States Government on beam weapons, and by the confusion between, on the one hand, the President's call for a total defence as a result of a long-term research programme and, on the other, the warmed-over silo defence which is being offered at least as a mid-term goal of the SDI.

Hecht does a good job of distinguishing between weapons and systems, noting the easy integration of target acquisition, aiming, fire and damage assessment in the eye, brain and arm of the human archer or marksman, as contrasted with a system in which these functions are dispersed over many satellites and centres of control and responsibility. He also covers well the technological side of the subject, describing the early high-power gas dynamic lasers, chemical lasers such as those which produce light from the reaction of hydrogen and fluorine, and excimer lasers now powered by electron beams. He emphasizes properly the degree to which plumbing and ducting is required for bringing the reacting gases to a chemical laser, but perhaps does not recognize adequately the degree to which similar considerations are important in venting gas from excimer lasers, because of the inevitable retention of some 90% of the pumping energy in the gas.

A further attractive feature of the book is Mr Hecht's tone of good-humoured scepticism, as in his observation that:

Pentagon officials have said that the Soviet effort [in laser weapons] is three to five times larger than the US program, an estimate that seems to hold constant even when the American budget is being sharply increased.

The treatment of the impact of space defence on national security is rather less solid. Hecht does not help to clarify matters by beginning with the "strategy of mutual assured destruction (MAD)" which, rather than being a strategy, is a fact of life. It is strategy to deter the opponent by threat of retaliation, but that one is able to do so and that one is deterred in return is a result of a technological fact — the destructiveness of nuclear weapons against societies — and not so much a matter of choice.

Strategic options flow from technological capability, and only to a certain extent can technology be created in the service of strategy. For instance, the technology suitable for defence against strategic ballistic missiles is for the most part even more suitable in the destruction of those same defences. Hence the ambivalence (almost to the point of schizophrenia) of those favouring space defence over the problem of where to base the defending weapons. As Hecht notes, Edward Teller explicitly recognizes that "it would be foolish to base a defensive system in space . . . because satellites are costly to put up and cheap to shoot down". A weapon that could destroy a booster in a few seconds might readily be destroyed by comparable weapons launched with the booster or already in space, and yet to keep weapons on the ground until the attack is in progress presents insuperable difficulties for intercept during boost phase. Mr Hecht dismisses such "pop-up defence" as unlikely even for mid-course intercept, but proponents of boost-phase defence and X-ray lasers argue valiantly that even a 180-s boost of an MX missile would be vulnerable to submarine-launched pop-up defence. Since the writing of this book, analysts have looked at countermeasures to some of the beam weapons, for instance fast-burn boosters to eliminate the threat of X-ray lasers or neutral-particle-beam weapons for boost-phase defence.

The issues surrounding beam weapons are at best murky and clear-thinking is not aided by statements of officials such as Dr George A. Keyworth, President Reagan's Science Adviser, who judges that:

. . . we can approach the debate about strategic defense as either optimists or pessimists and still come to the same conclusion: We must proceed. The optimistic view would have the United States be the first to develop effective defensive capabilities, giving us a persuasive negotiating posture for arms reductions . . . the pessimistic view sees the Soviets as the first to develop a real defensive capability . . . [*Aerospace America*, April 1984].

Whatever the persuasive power of this argument, it has no foundation in logic:

what about those technologists whose optimism is focused on penetration aids for defeating defences? No one doubts that starting from a conclusion one can find (some) arguments to support Keyworth's view, but in a democracy, and particularly when the questions involve not only hundreds of billions of dollars but the survival of nations, we need all the strength that open discussion and decision-making can provide. *Beam Weapons* will help to that end, together with the more extensive and technical *Space-Based Missile Defense* of March 1984 by the Union of Concerned Scientists, and especially the Background Paper by Ashton Carter, published in April 1984 by the Office of Technology Assessment.

In the book, Hecht displays a degree of constructive optimism and cites two possibly beneficial outcomes from beam weapons. One is "some sort of international peace-keeping organization to operate defensive satellites". The other is the hope that beam weapons "would tip the strategic balance of power to the defense", in providing missile defence, and "if the great powers can cope with the challenge of a transition to a strategic defense, the result could be a stabler world". I, however, would agree with the position taken by the French government in its presentation earlier this year (12 June) to the Committee on Disarmament in Geneva:

A situation in which each of the two principal powers sought to render its territory totally invulnerable, that is to say to escape any retaliation, without, however, being certain of being able to achieve this invulnerability, is fraught with danger.

On the one hand, the announcement itself of the intention to proceed with the creation of such systems constitutes itself an incitement to the acceleration of the offensive arms race: each power seeks to overcome (by saturation) the BMD [ballistic missile defence] systems envisaged by the other party, and in addition to increase greatly the number of non-ballistic carriers of nuclear weapons — notably cruise missiles.

Mr Hecht himself notes that the most credible role for a pop-up X-ray laser "is as part of a first-strike system, which would be put in place before the attack to defend against any enemy missiles which survived a massive first strike".

Beam Weapons is marred by misprints and by an irritating amount of repetition. Some references are quoted inaccurately, and the simple calculations provided would have been even more helpful had the arithmetical errors been corrected. But taken as a whole the book is a fair, readable attempt to make some sense out of the confusing claims and counterclaims about the feasibility and utility of directed energy in warfare. □

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Spectroscopy of novel systems

From R.C. Thompson

Atomic spectroscopy is alive and well thanks to the continuing development of novel experimental techniques and their application to new areas of interest.

THOSE people who think that the subject of atomic spectroscopy was wrapped up several decades ago would have seen much evidence to the contrary at the recent EGAS conference held at Imperial College¹. Completely new areas of spectroscopy are still arising, bringing with them new and interesting physics. For example, the study of Rydberg atoms (atoms where one electron is excited into a state of high principal quantum number) has blossomed recently, and an up-to-date review of this field was given by C. Fabre (Ecole Normale Supérieure, Paris). Because many atomic properties scale as large powers of the effective principal quantum number n^* , Rydberg atoms can have large diameters (up to a fraction of a micrometre — large enough to be measured using a fine sieve), very long lifetimes and large polarizabilities. These facts make possible very unusual experiments — the detection of single microwave photons using Rydberg atoms as sensitive detectors; the modification of the spontaneous decay rate of atoms by putting them into a high-Q resonant microwave cavity; and even the possibility of constructing a single-atom laser (the usual many-body nature of laser action is made possible by the interaction of the atom with its images in the walls of the cavity). In experiments where a thermionic diode is used to detect ions formed by collisional ionization of laser excited Rydberg atoms, transitions to states with n up to 200 have been studied.

A relatively new area of spectroscopy was reviewed by G. Soff (Gesellschaft für Schwerionen, Darmstadt). Super-heavy quasi atoms are created for very short periods (10^{-20} s) in collisions between heavy projectile and target nuclei at energies of a few MeV per nucleon. As an example, at a total nuclear charge $Z = 164$ (formed in a Pb + Pb collision), the nucleus created has a radius of 10 fm, while the radius of the 1s electron wavefunction is 20 fm. Some unusual physical effects occur under such extreme conditions, such as the spontaneous emission of positrons during the collisions. This becomes energetically favourable at a combined charge $Z > 173$, when the 1s state enters the lower continuum of the Dirac Hamiltonian, that is, the binding energy becomes greater than the energy required for creation of an electron-positron pair. Measurements of

this and other effects in progress at GSI enable tests to be made of quantum electrodynamics in novel situations.

Another new development is the first observation of photon correlations in a true second-order two-photon decay (H.J. Duncan, University of Stirling). In this experiment, metastable hydrogen atoms (in the 2S state) in an atomic beam decay emitting two photons in random directions. Events are recorded where one photon enters each of two detectors placed 180° apart. Measurements of both the linear and circular polarization correlations between the two photons enable a test to be made of the celebrated Bell's inequality, which distinguishes the predictions of quantum mechanics from those of local realistic theories. As in other complementary experiments performed recently, these results favour quantum mechanics, but the controversy about whether or not quantum mechanics provides a complete picture still rages.

One of the highlights of the conference was the screening of a film showing the results of a series of calculations of the behaviour of an atom being irradiated by laser light (B.W. Shore, Imperial College London and Lawrence Livermore National Laboratory). The idealized two-level atom is a familiar concept, but here a slightly less idealized ten-level atom is treated, the results being displayed in the film as a two-dimensional plot of the density matrix of the atom as a function of

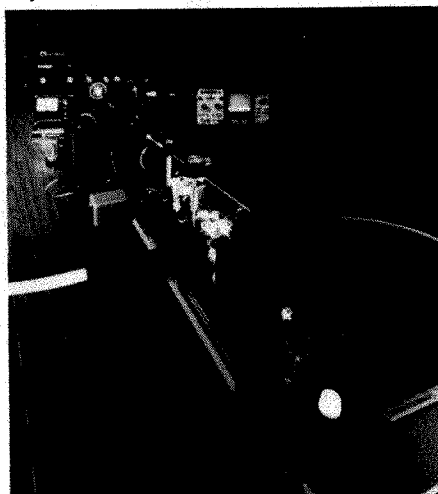
time. The phenomena illustrated include the periodic complete population inversion obtained with resonant coherent excitation and the gradual buildup of population in a specific excited level when the laser frequency corresponds to a multiphoton resonance. The film gave a valuable insight into the physical processes described by sets of equations treated usually only in an abstract mathematical manner.

Experiments using laser excitation were prominent at the conference and one striking example of the way the use of laser techniques is still growing is the application of coherent radiation (at wavelengths below 100 nm) generated by four-wave mixing in Kr gas to the study of VUV transitions in Xe, including the measurement of absolute wavelengths to 2 parts in 10^6 (T.J. McIlrath, University of Maryland and National Bureau of Standards, Washington).

Most laser experiments, however, are performed in more conventional wavelength ranges. The availability of reliable single-mode tunable dye lasers covering the whole of the visible spectrum has meant that high resolution and power are now easily achieved in this region. Numerous Doppler-free laser techniques have been developed for the recording of spectral lines under many different source conditions; the laser has thus become an invaluable aid to the study of the structure of atoms and molecules.

A recent trend is for much atomic spectroscopy to be performed at large centralized facilities such as accelerators (used for example for the production of beams of atoms in various states of ionization) and nuclear reactors (for the creation of rare isotopes). This places more stringent requirements on the reliability of experimental apparatus, so that there has been a move in general towards computer controlled equipment and automated data acquisition. The use of computers also in the analysis of data has meant that more complex atomic systems can be investigated and this complements the theoretician's growing ability to perform calculations on such systems.

The EGAS conference demonstrated the broad range of the subject, with sessions covering topics as diverse as term analysis, isotope shifts, electron spectroscopy and collisional broadening.



The Coherent 699-29 Autoscan, a self contained dye laser spectrometer ideally suited to high-resolution absorption spectroscopy. The heart of the system is the Coherent 699-21 actively stabilized single-mode ring dye laser. Control is provided by an Apple II microcomputer, which can also be used for up to three channels of data acquisition. Circle no. 100 for further details.

R.C. Thompson is at the Division of Mechanical and Optical Metrology, National Physical Laboratory, Queens Road, Teddington, Middlesex TW11 0LW, UK.

¹ The Sixteenth European Group for Atomic Spectroscopy Conference was held at Imperial College, London on 10-13 July 1984.

Spectroscopy and spectrometry

Automation and computer-links are the main themes among the latest in spectrophotometers and spectrometers

● The CPS-240 six-cell positioner from Shimadzu enables the Shimadzu UV-240 spectrophotometer to display absorbance in a bar graph format, plus data as difference in absorbance multiplied by a factor for all or part number of the six cells. Also available is the capability to look at different wavelengths at each of the six cells and change the absorbance range for each. Temperature can be controlled over the range 16°–60°C, and a manual determination of which cell is being looked at is possible at any time.

Circle No. 101 on Reader Service Card.

● A new economical system from Pye Unicam allows absorbance calibration for spectrophotometers in the ultraviolet and visible ranges. The set of four neutral-density filters provides a comprehensive check of absorbance linearity up to 2Å. Pye Unicam's original set of nine filters, calibrated up to 3Å, is still available for users needing a fully comprehensive check of absorbance linearity across the whole intensity range.

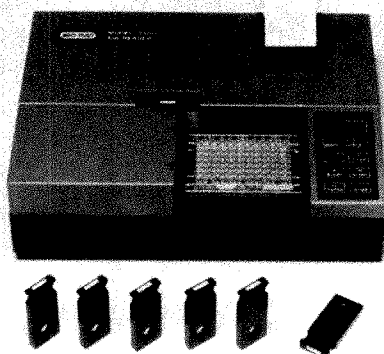
Circle No. 102 on Reader Service Card.

● The computerized D.B. scanning spectrophotometer UVIDEC-610/650 from Jasco International now incorporates software modifications to simplify routine analysis and enhance data presentation. A battery-backed memory stores data for at least two years, enabling users to save pre-selected parameter combinations for each of the six main methods already programmed into the computer. In addition, up to five sets of parameter/command instructions can be stored, so that routine or repetitive analysis may be performed at the touch of a key. The cathode-ray tube display includes a 'screen edit' function whereby users' comments are entered through the keyboard, stored in memory, and printed out as an integral part of the final report along with spectra and all other relevant data. Newly available computer accessories include double disk drive for data/spectra storage, and a full QUERTY keyboard for preparation of specific application programmes in BASIC or FORTRAN.

Circle No. 103 on Reader Service Card.

● Newly introduced onto the US market, the low-cost infrared data station from Heyden Data Systems of North London — Spectrafile IR — is based on the Apple microcomputer. Spectrafile IR consists of three main modules: data acquisition and instrument control, data analysis and spectral search. The system provides operators with full control over an IR spectrophotometer with individual interfacing included in the system price.

Circle No. 104 on Reader Service Card.



The Bio-Rad photometer for reading microtitration plates.

● The Bio-Rad Model 2550 EIA reader is a photometer that automatically reads, with blanking and hard copy printout, a 96-well microtitration plate in less than 60 seconds. A digital absorbance-range display and plate-position indicator, as well as an operator error indicator and an absorbance overflow indicator, guide the user. Four high quality, narrow bandwidth interference filters, 405, 414, 450 and 492 nm, are supplied as standard equipment. Optional filters permit wavelength measurement at 380 to 700 nm. The reader has two measuring modes. The absorbance mode provides direct hard-copy printout of the absorbance values to three decimal places and matrix mode makes it possible to preselect an absorbance range, and automatically divide it into ten equal segments. The microprocessor assigns a value of 0–9 for each segment, and assay absorbance readings are catalogued into the appropriate range segment. All values are printed out in an 8×12 matrix that corresponds exactly to the layout of the 96-well microtitration plate.

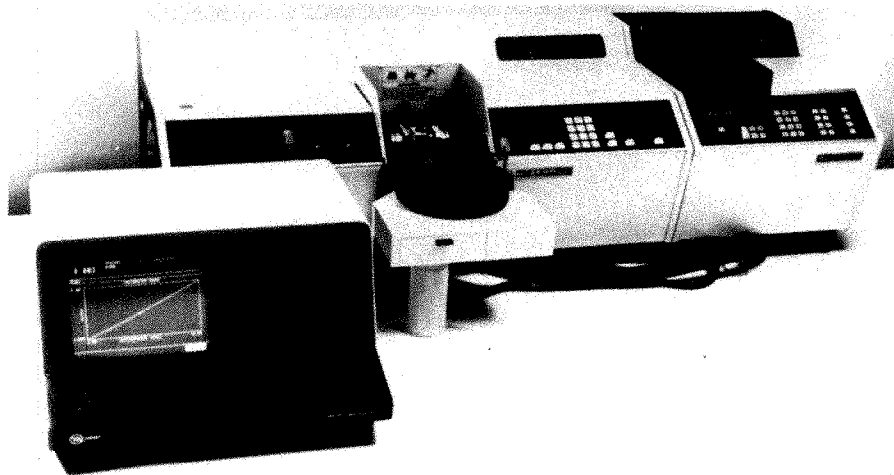
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● The new range of Fourier transform spectrometers from Bomem, Inc. extends the operating wavelength range from the ultraviolet to the far infrared. A standard spectrometer with suitable detectors can produce spectra from 50,000 cm⁻¹ to 4 cm⁻¹. In addition to a UV to far-IR capability, the new spectrometers feature a fully evacuable spectrometer to eliminate water vapour; a five port sample compartment selectable from the control computer, and an automatic optical alignment system that ensures the system is always at peak efficiency. Bomem systems are available with a choice of resolutions, up to a maximum of 0.002 cm⁻¹.

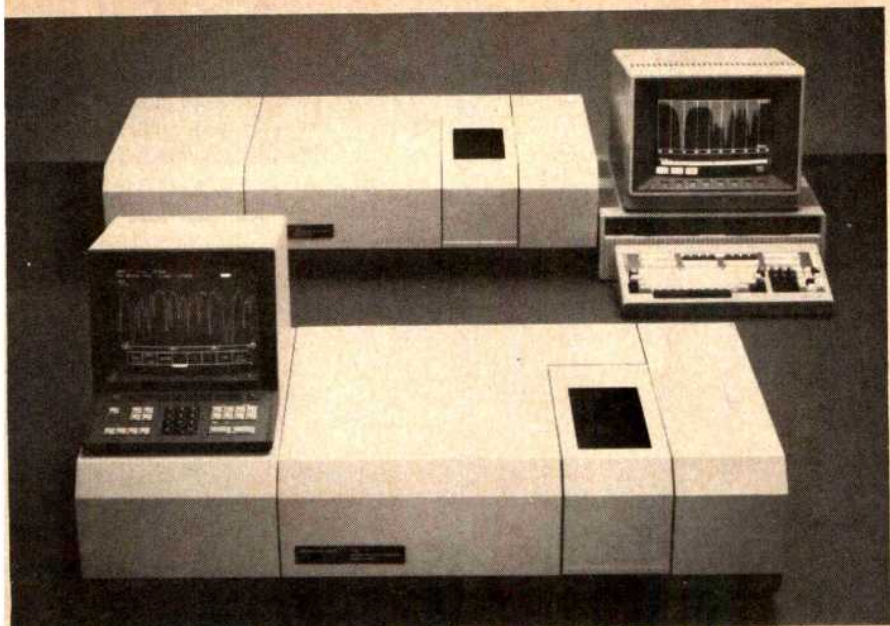
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● By using a new software package, the Varian DS-15 computer can be linked to an AA-1275 or AA-1475 spectrophotometer for complete atomic absorption data management. The DS-15 is a powerful desk-top computer with 12-inch CRT, 128 Kbytes RAM and two disk drives providing 720 Kbytes of storage. The single-beam AA-1275 and double-beam AA-1475 atomic absorption spectrophotometers are brought together by the new Varian software in a package specifically tailored to meet the needs of the busy analytical laboratory. No programming experience or typing ability is required. The operator is guided through a series of simple selections using menus and soft keys. Results are collected automatically when flame or furnace autosamplers are used but samples may also be presented manually. Flexible report generation allows reports to be tailored to individual needs. The operator may choose the amount of data to be printed and the format of the report. Data from flame, furnace and hydride analyses may be combined in a single report.

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Varian's AA-1475 spectrophotometer linked to the DS-15 data station.



The 1700 Series Fourier transform infrared spectrometer from Perkin-Elmer.

● Based on a single high-performance interferometer, Perkin-Elmer's 1700 Series of Fourier transform infrared spectrometers is a new range of compact, mid-infrared instrumentation. The instruments in the 1700 Series differ in their data handling capabilities. The spectrometer itself uses an improved Michelson interferometer designed to compensate internally for any effects of dynamic misalignment, providing an extremely stable system resistant to vibration and disturbances during a scan. Interferometer alignment is very simply carried out by a closed-loop servo system and is initiated from the control console. The standard spectrometer has a frequency range of $4,400\text{ cm}^{-1}$ to 400 cm^{-1} and a choice of resolution down to 2.0 cm^{-1} . Resolution can be increased to 1.0 cm^{-1} by the addition of an optional memory board.

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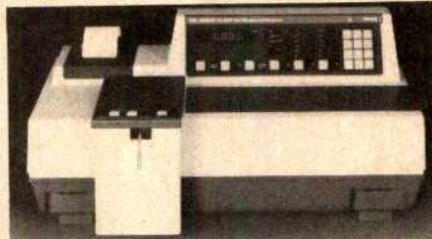
● The new GN Series NMR spectrometers from General Electric incorporate a 293D pulse programmer along with the GN series data system and NMR software which enables the user to program a wide variety of pulse sequences. This feature, together with the ability of the instrument to vary phase shifts from 0 to 360° , makes it possible to perform most types of multi-pulse, multiple-quantum NMR experiment. In addition, the programmable pulse modulation and phase shift capabilities of the GN Series also make it possible to perform all the 2-D NMR experiments currently in use. The GN Series system incorporates an easy-to-use multiple-level, menu-driven software package which includes automatic shimming, locking, gain, spectral phasing and integration programs.

Circle No. 109 on Reader Service Card.

These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.

● The new Nicolet 20DXB and 20SXB FT-IR spectrometers are based on field-proven Nicolet FT-IR technology. The 20B Series performs with a maximum resolution of 0.5 cm^{-1} . Resolution is continuously selectable from 0.5 cm^{-1} to 64 cm^{-1} . The spectrometer can scan at rates as fast as 4 per second, making it suitable for monitoring dynamic processes such as capillary GC-IR, catalysis, and polymer curing. An external collimated beam is standard for 20SXB GC-IR interface and remote detector experiments. A computer-controlled flip mirror allows fast, easy, precisely pre-aligned switching between the two sample path geometries. Designed for the chromatographer, the new 20SXB GC-IR software provides simplified, automated operation, from real-time data collection and display to identification of components.

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For industrial and teaching laboratories, the Pye-Unicam PU8600 spectrophotometer.

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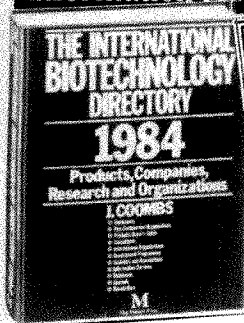
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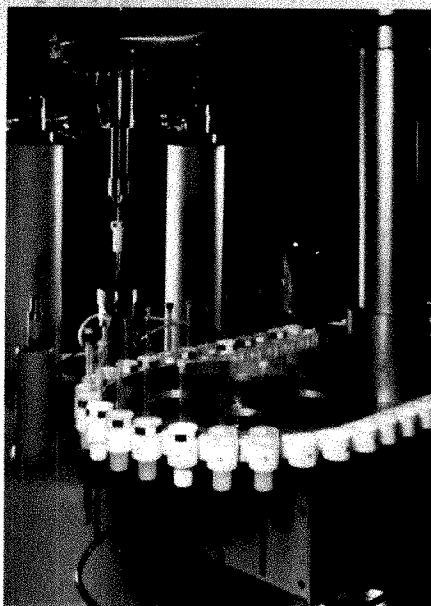
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• Developed from the successful PW 1400 series, the PW 1404 sequential X-ray spectrometer from Philips Analytical features multilayer 'crystals' giving enhanced sensitivity to light elements — down to C and B. The choice of side-window X-ray tubes has also been extended to include a new dual-anode tube that allows the use of a single X-ray source without compromises over a wide range of applications. A compact, high-efficiency generator is built into the spectrometer and other features include an auxiliary high resolution collimator and programmable channel masks that reduce background.

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• Spectrafile-UV is an ultra-violet spectrophotometer data station which collects, plots and manipulates UV data. Features include full instrumental set-up and control. Spectrafile-UV allows data to be collected and displayed and stored on disk. Data can be manipulated with the functions: add, subtract, multiply, integrate, differentiate, normalize, smooth and others. Customized routines for kinetic studies are also available and Spectrafile-UV, from Heyden Datasystems, interfaces to any UV/VIS spectrometer.

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• Sirius 100 and Cygnus 25 are FTIR spectrometers from Mattson Instruments Inc. They offer resolution capabilities of 0.125 wavenumbers and 0.5 wavenumbers respectively over the mid-infrared spectral range with optional range extensions to 25,000 wavenumbers and 10 wavenumbers. Notable features of both spectrometers are high-throughput 2-inch diameter optics, a Michelson interferometer based on cube-corner retroreflectors for high dynamic stability (no air bearings), and a large accessible sampler chamber.

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• The STARLAB data system available from Glen Creston of Stanmore, Middlesex, is a UNIX-based software system with multi-user/multi-tasking capability and 32-bit arithmetic. Data storage capacities from 10Mb to 340Mb are provided by Winchester disks, and back-up storage for data and software by 8-inch floppy disks is standard. Spectrometer operation and data manipulations are commanded through a standard graphics terminal.

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• The Corning Gilford Response range of UV/VIS spectrophotometers has now been expanded to encompass three separate models. All feature an autoranging photometer, transverse sample compartment, high resolution graphics, data storage facilities and a 16-bit resident computer. The basic Response is supplied complete with a single cell holder and programs for wavelengths scanning, kinetics, time scanning, multi-wavelength analysis, standard curves and sample read. The second unit has an automatic six-position cell holder as standard and has the capability of adding gel scanning, rapid sampling and a six-position thermostet cuvette holder for electronic control of sample temperature. The most advanced Response has two disk drives for data storage and recall, with extended software and applications.

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• The Dynatech MR600 automatic microplate reader automates both new and established techniques requiring accurate optical determinations of end-points. Covering the visible and UV range and utilizing the popular 96-well format microtitre plate, MR600 offers miniaturization and ease of handling in numbers. When linked to a microcomputer the MR600 becomes a powerful spectrophotometer.

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Helping out in Bhopal

It would be easy to draw the wrong conclusions from last week's harrowing events at Bhopal's Union Carbide plant.

BHOPAL in Mahdya Pradesh in central India is a long way from Danbury, Connecticut, where Union Carbide has its headquarters. In neat New England, everybody knows that it is difficult to win permission to build any kind of chemical plant within sight of human habitation and that, thereafter, zoning laws will ensure that the surroundings are not promptly occupied by people. From a vantage point in New England, it is not easy always to remember that, in India, people will instead gravitate towards shanty structures as close as possible to the gates of whatever factories there may be. How else to get to work?

So there is a sense in which the worst disaster so far caused by the chemical industry, at least since the destruction of the port of Halifax, Nova Scotia, in 1917, springs from the circumstance that the plant had been built in India. Two other Indian features of the tragedy stand out. First, the people working at the Bhopal plant seem to have been slow to appreciate the scale of the danger. Second, the anguish vividly carried round the world by the television cameras seems not to have matured into the anger, even hysteria, there would have been had the accident occurred on the edge of a European city — or in Connecticut. But to write off this stocism as a manifestation of Indian fatalism, or familiarity with death, would be greatly to demean the responsibility with which both the community of Bhopal and the authorities of the state and of the centre in New Delhi have behaved. That this should have been possible in the thick of an election campaign is doubly remarkable.

None of this guarantees that the affair will pass off without trouble for Union Carbide, or for the operators of other chemical plants in India and elsewhere in the developing world. The contingency lawyers will see to that. But as yet, everybody recognizes, it is too soon to apportion blame. That is what the inquiry now under way is for. Whatever the findings, however, one source of confusion stands out. Although the plant at Bhopal seems to be a carbon copy of one operated in West Virginia, Union Carbide owns only a narrow majority of the Indian operating company, with the remainder in the hands of Indian interests according to the formula by which India seeks to blunt the impact of overseas investment. So Union Carbide may be expected to shoulder only 51 per cent of whatever blame is found to lie at the door of the plant's owners and operators. Legal caution would therefore suggest that the company should await the outcome of the inquiry before deciding what to do, and that it should then await the outcome of whatever procedural niceties are required to determine its responsibilities, if any.

Urgency

The implied delay, perhaps for several years, would be a crying shame. The need for relief, and for arrangements to be set in hand to care for the victims of this tragedy, is urgent. Now is when the anxiety of those injured but not killed will be greatest, and when fears of an uncertain future will most prey on people's minds. Without prejudice, as the lawyers are forever saying, which in this case means that the US parent company may be strictly blameless, Union Carbide should make a quick start on the provision of the resources to ensure the medical services that will be needed for

decades ahead to care for the people exposed to methyl isocyanate ten days ago. If the company considers the task beyond its own resources (which is possible) it should cajole other Western corporations similarly at risk in India to share the burden. It is not too much to ask that the governments concerned should also help. The objective at this stage should simply be to put in place the facilities that Indian medicine will need to monitor the health of those who have been exposed, and to provide what treatment is possible. In this instance to act swiftly is more important than to act prudently.

But would not such an initiative imply an admission that blame applies? No, is the simple answer. The lawyers' *caveat* is full of meaning. And when was a proper sense of compassion ever blameworthy? But if India proudly insists that it wants not compassion (with all its neo-colonialist connotations), but only justice, this is nevertheless an occasion when compassion should be offered. Catastrophes on the scale of Bhopal are too great, and their implications too ramified, to be handled alone even by the wealthiest societies. And this is unlikely to be the last accident to occur with the dreadful character of what has happened at Bhopal.

Other superficial interpretations of what has happened also cry out for denial. Outside India, there has already been a wave of soothsaying to the effect that what happened at Bhopal shows that the "export" of advanced technology to developing countries is in some sense inappropriate. That is the true neo-colonialism. And as an opinion, it is clearly false. For one thing, India is far from being technologically innocent. Moreover, the indigenous chemical industry is as strong as any other Indian technology. But there is also a home-grown nuclear industry (whose plants have been managed successfully if, perhaps, less well than they might have been) not to mention a thriving indigenous space enterprise with its own satellites that will, in due course, also be launched indigenously. There is no sense in which a sophisticated plant for manufacturing pesticides is inherently out of place in India.

Interdependence

What is wrong with Indian technology is not the amount of it or even the state of development but that it is too thinly spread. For at least the past three decades, India has followed a policy of development on a broad front, on the grounds that only by attempting everything (and, in practice, doing a little of everything) would it be possible to shake off the colonial yoke. The result is that India does everything less well than superbly, and at least a little late. A better strategy would be to do some things so well that their products could be sold on the export markets, earning the foreign exchange with which to buy the products of the technologies left temporarily on one side. This is how other economic systems, most of those in Western Europe included, set out to match their capabilities against the complexity of modern technology. They have discovered that economic interdependence does not mean thralldom. Why should India be all that different? But the consequence, at Bhopal, would either have been that the methyl isocyanate plant would have been but one in

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a larger and stronger indigenous industry or that it would not have been there at all, but perhaps in Connecticut.

The doctrine of self-reliance (as distinct from self-sufficiency) was always due to be an election issue. Now, after Bhopal, Mr Rajiv Gandhi will find himself also under pressure from the traditionalists who argue that India's error is to have aimed at something more ambitious than being a village economy. He can be relied upon to resist that claim, on the sure grounds that there is no other way than technology to meet the aspirations of the villagers. For has not the new agricultural technology, he can say, made them well-fed for the first time in history?

But on past form, Mr Gandhi will not turn his back on self-reliance. The two weeks remaining of the election campaign is not the time for changing tack, but afterwards there is the strongest case for looking again at this policy, seeking some way of concentrating resources on a field in which India could make a stronger mark.

Inappropriate

The other common false lesson drawn from what happened last week is that modern technology may as such be inappropriate, not merely in India but everywhere. People say "Look at Three Mile Island", intending to imply that the two accidents are similar and that the technologies that made them possible should ideally be avoided. In reality, nothing could or need be further from the truth. Plants for making pesticides of various kinds have operated safely and successfully during the past half-century; only some of them are risky, as these things go. (One of the ironies of the Bhopal accident is that the pesticide being manufactured, Sevin, is a substitute for DDT introduced in an attempt to avoid the risks of that organochlorine compound; the process for manufacturing the new pesticide has already done more damage than DDT.) But it is a good question whether large quantities of a dangerous chemical, particularly if it is an intermediate, should ever be stored as they were in India. Carrying these chemicals about the country (as is done in the United States) is also a needless risk. What needs to be learned from last week's accident in technically advanced societies is that the handling of novel chemical processes needs constant reassessment. □

Research charade

The UK research community should respond forcefully to the latest disappointments.

THE feckless mishandling of the British research enterprise by the British government has now been invested with an element of farce. So much is plain from last week's events, beginning with the government's attempts to limit the political damage done by Sir Keith Joseph's plan to make higher education more expensive for the student offspring of the well-to-do (see *Nature* 6 December, p.483). Part of the plan to contain the damage was the accelerated publication of the document describing how £14 million of the funds thus "saved" would be used to make the British research councils less constrained. The chairman of the Advisory Board for the Research Councils (ABRC) dutifully stood up in public and said how valuable the extra money would be. But by the following day, Sir Keith Joseph had been forced by the House of Commons to go back on his proposals (see page 582) and by the Treasury to claw back £4 million of what he had been offering a few days earlier. The University Grants Committee, less quick off the mark, similarly had £6 million snatched back from the £10 million it had been offered for universities in crying need of new equipment, but without the added humiliation of having advertised its gratitude in public.

What this pantomime has done is to make a monkey of those who administer the British government's support of civil science, and of government policy on research in general. The common complaint by the research councils in recent years that they have not known where they stand from one year to the next has now been overtaken by the simple truth that they may not know from day to day. The simplest reason why all this is a nonsense is that if

the government was right, at the beginning of last week, to have taken the view that the need for extra funds for science had become so urgent as to justify the political risks of increasing higher education costs, it cannot have become, by the end of the week, proper for it to say that it would let the needs of research go hang.

Even those who believe the British government is right to insist that public expenditure should be tightly controlled will think it humbug that the government pretends that nowhere in its budget of £125,000 million is to be found £10 million that might be spared for the support of research. Indeed, the sum concerned is smaller than the errors that arise in estimating the various components of British public expenditure.

Ministers at other spending departments than the Department of Education and Science will now be quick to say that Joseph's muddle is his own affair, that he should have fought harder, some months ago, when next year's budget was being hammered out and that he should not now expect them to save him from the trouble he has brought down on himself. What, in all this, has happened to the doctrine of collective cabinet responsibility by which British governments set such store, especially when they need to make some recalcitrant minister toe the party line?

Several things should happen now, at least one of which should be a ritual resignation. Ordinarily, it would have been Sir Keith Joseph who would have packed his bags, but he and his government appear to have agreed that that may not be necessary. The members of ABRC and the heads of the research councils are differently placed. Most of them are, in any case, people with other and even better things to do, and cannot much profit from having been made to look like fools. To walk out at this stage would not bring the administration of science to a halt. To stay will be to let the government continue to behave as if the noise there has been about the condition of the British research enterprise is a ramp conceived by self-interested people eager to take for themselves and their cronies a larger share of the public pie, mostly for making their ivory towers more comfortable. Resignations after events such as those at last week can have no immediate effect. Their value is that they would show that people mean what they have been saying about the sombre condition of the research enterprise in Britain.

Initiative

For the rest, the British research community should resolve to seize the initiative for the management of its own affairs that will be there to seize in the period of embarrassment that is bound to follow. It will also be politically impossible to go back on the Prime Minister's promise five years ago that the science budget will be protected, which appears to mean that it will be indexed against inflation and then made to accommodate a variety of needs that were not apparent at the beginning. The best strategy now is that already being followed by two of the research councils (for science and the natural environment), which have embarked on redefinitions of their budgets so as to enhance their flexibility. The Agricultural and Food Research Council has already moved along that road. The need now is that this process should be radical, based on the assumption that the government, the research councils' paymaster, which is plainly indifferent to their present plight, will be equally indifferent to the ways in which they organize their affairs.

ABRC has a special role to play. It is the only committee on which the University Grants Committee and the research councils are both represented. It is also a means by which the British academic community as a whole could be consulted. To salvage the self-respect damaged by last week's events, ABRC should begin to throw its weight about more vigorously, even raising in public some of the questions about British research which are ordinarily considered taboo. Does it, for example, make sense that the British government should administer civil science by the account-book simply because that expenditure is identifiable and thus easily controlled, while allowing the vast apparatus of defence research and development to go its own way because it happens to be part of an even larger enterprise? □

Toxic gas

Pesticide plant leak wrecks disaster in India

New Delhi

THE leak of toxic gas from the Union Carbide pesticide plant in the central Indian town of Bhopal on 3 December has left at least 2,500 people dead and killed hundreds of cattle. The death toll is likely to increase, with thousands still in hospital three days after the disaster, which affected 200,000 people living within a 7-km radius of the plant. The cause of the leak is being investigated by chemicals experts with the help of a team flown in by the US multinational company. Meanwhile, the state government of Madhya Pradesh has arrested six workers, including the plant manager, ordered indefinite closure of the plant and started a judicial inquiry. The Central Bureau of Investigations is looking into the possibility of sabotage. It seems likely that Union Carbide India Limited

(UCIL), the wholly-owned US subsidiary, will be faced with large claims for damages.

The killer gas was methyl isocyanate (MIC), used in the plant at Bhopal as an intermediary in the manufacture of some pesticides, including Sevin, which is widely used. According to a UCIL spokesman, the leak occurred in one of three underground tanks storing MIC, which is liquid up to 38°C and extremely volatile. He said the vent caustic soda scrubbers provided for neutralizing accidental releases failed to cope with an excessive pressure build-up. The tank held 45 tons at the time of the leak. It is not known how much gas escaped from the plant which, flouting existing safety guidelines, is just 1 km from the railway station and 5 km from the centre of town. UCIL said that the leak was plugged 45 minutes after it was discovered, but by that time much of the town of Bhopal had been virtually turned into a gas chamber, and was a vivid demonstration of what chemical warfare would be like.

The UCIL plant, commissioned in 1977, employs 800 workers. Ironically, the Indian government only last year extended

the plant's licence for seven years after a promise from UCIL that it would secure from its parent US company the technology to handle "emergency situations like toxic gas release, sometimes accompanied with fire, endangering the safety of the community". There had been two earlier minor incidents in the Bhopal plant, one in 1981 when a leak from a phosgene storage tank killed several workers; in the latest incident, oddly enough, only one worker was injured.

In the nightmarish aftermath of Bhopal, the Indian incident is bound to become a factor in the forthcoming general election. The opposition is critical of India's policy of importing hazardous technology and pesticide licensing. The recent collaboration with Monsanto Corporation of the United States for manufacture of the herbicide butachlor, which is banned in the United States, is under attack. But pesticides made using indigenous technology in India are produced under lower safety conditions than those that existed at Bhopal, according to a spokesman for the Ministry of Chemicals. Six companies in India have been licensed to make MIC and several small pesticide manufacturers are transporting phosgene in bottles. Following the Bhopal tragedy, safety has been tightened at the state-owned Indian Petrochemicals Limited in Baroda which produces hydrocyanic acid, which is far more deadly than MIC.

K.S. Jayaraman

Ways of death

Washington

RELATIVELY little is known of the toxicological effects of methyl isocyanate, which is produced by reacting methylamine and phosgene and reacted with α -naphthol to produce the pesticide Sevin. Its strong primary irritant properties probably accounted for the majority of deaths: conversion to cyanide is not thought to occur at a significant rate *in vivo*. The US Occupational Safety and Health Administration limits exposure of workers to 0.02 parts per million over an 8-hour period. Tests on rats and on human volunteers indicate that the chemical's most immediate effects are upon the respiratory tract and the cornea; in rats, the cause of death was a massive build-up of fluid in the lungs. In humans, exposure to 2 parts per million caused nose and throat irritation, 21 parts per million a suffocating effect and severe irritation of the respiratory tract and eyes.

The cause of death after acute exposure would probably be either bronchospasm or what amounts to drowning in released body fluids. Failing this, death could result from the secondary effects of loss of blood oxygen and acidosis, or from congestive heart failure. According to Dr Trent Lewis of the National Institute of Occupational Safety and Health, survivors will probably suffer increased incidence of pulmonary fibrosis, emphysema and chronic bronchitis. The respiratory tract may become hyperreactive to a variety of irritants, and changes in lung architecture may increase susceptibility to infectious pneumonia. Necrotic lesions of the cornea may lead to permanent blindness or impaired vision.

Tim Beardsley

UK government review

More bad news for science

BRITISH government expenditure in basic science is due to fall by three per cent (at constant prices) over the next two years, according to the second *Annual Review of Government-Funded Research and Development* (HMSO, £7.50) published on Tuesday. Prepared by the department of the Chief Scientific Adviser at the Cabinet Office, Dr Robin Nicholson, this is a second and more accurate shot at determining the trends and distribution of government science spending.

The figures were prepared too late to include the recent goings-on at the Department of Education and Science (see p.582), but they do add some grist to the argument that science in Britain is threatened. Compared with 1983-84 expenditure, and in real terms, the Cabinet Office expected the Economic and Social Research Council budget to have fallen to 84 per cent of its original value by 1986-87; Agricultural and Food to 91 per cent; Medical to 94 per cent; Natural Environment to 98 per cent; and Science and Engineering to have risen to a princely 101 per cent.

In civil applied research ("expenditure to improve technology") things look only slightly better. The total government expenditure in this area will be £807 million in 1986-87, the Cabinet Office estimates, some 9 per cent greater in real terms than in

1983-84. But this represents a decrease from the current year (1984-85), which rides at 15 per cent above last year. The biggest projected declines to 1986-87 compared with the present year come in the Department of Energy (down 7 per cent), the Department of Environment (down 9 per cent) and significantly (as it spends half the government budget in this area) the Department of Trade and Industry (down 8 per cent).

Defence research and development, however, will be maintained at constant prices to 1986-87, although even these large sums (at £7,077 million in 1984-85 some 50.3 per cent of all government-funded research and development) are falling as a fraction of defence procurement.

The Cabinet Office also takes a special sectoral look in its report at British spending on marine science, in an attempt to identify "gaps and overlaps" in spending; but here it prepares the ground, unfortunately, for more cuts. "There seems cause for considering" write the authors "whether the present provision of facilities [15 towing tanks, 7 cavitation tunnels, etc.] is excessive for a shipbuilding industry which declined 30 per cent between 1975 and 1981". Decline, it seems, begets decline.

Robert Walgate

UK science

Now you see it, now you don't

A MUCH-needed present for British science (generally regarded as being on its uppers) was divided and praised last Tuesday morning — but taken away, at least partially, in the afternoon. The moral of this strange story is that Britain's mechanism for allocating budgets between ministries is in need of drastic overhaul.

At noon on Tuesday, Sir David Phillips, the chairman of the Advisory Board for the Research Councils (ABRC), which each year allocates monies to its member councils, was praising the "courageous" action of Secretary of State for Education and Science Sir Keith Joseph in raising new money for science in 1985–86 and subsequent years. (He had promised an effective £16 million annually for the research councils by a reallocation of resources within his own cash-limited ministry, making well-off British parents pay a little more for their children's university education and giving this as a bonus to science.)

But by 3 p.m. the same day, Sir Keith was having to bow before a storm of protest in the House of Commons from what sometimes seems to be the only effective British political opposition, given the large Tory majority — the Tory back-benches.

Back-benchers had reacted with horror at what was really a fairly moderate increase in parental contributions to student grants among better-off families (see below). Sir Keith hung on to his principles (there will be a commission on student funding), reduced the new burden on parents, and was partly bailed out by the Treasury. The result was some £3 million less than expected for ABRC next year (and £6 million less for the "equipment grant" of the University Grants Committee). It was a "very disappointing" result according to a spokesman for one of ABRC's member councils, the Medical Research Council (MRC).

Science will, however, still get more than was promised before Sir Keith's grants saga began. The way the Department of Education and Science now explains the figures is this. Sir Keith's plan had been to eliminate the minimum student grant of £205 a year, to increase the parental maintenance contributions for family incomes above £11,500 and to impose tuition fees for incomes above £20,000. This would have released £60 million in a full year but only £39 million next (not a full year as the financial and academic years do not

match). Sir Keith has now been forced to cancel the imposition of tuition fees. This loses his department £21 million next financial year. The Treasury has offered £10 million, leaving £11 million to find within the department.

The effect on the research councils is a £3 million loss compared with expectations at noon on Tuesday. In the financial year 1985–86, ABRC was to have received £571 million (an increase on last year based on price indexation) plus a bonus from Sir Keith's grants plan of effectively £14 million (plus £2 million from a salaries adjustment). This bonus will be reduced to £11 million (plus the £2 million). An intended £10 million bonus on the UGC equipment grant is similarly lopped by £6 million, and £2 million is lost elsewhere.

The cut (and the bonus) may seem small, but on the original plans ABRC was to spend £2.75 million of the bonus on "restructuring" (effectively, pensions and redundancy). So the bonus amount for science is really falling from £13.25 million to £10.25 million, a large enough fraction that the horse trading over how to divide up the money among the research councils must begin again.

Discussions on this are taking place this week, when, unfortunately for the Science and Engineering Research Council (SERC), its chairman, Professor John Kingman, out of the country. Last week he had been describing his pleasure at the "enormously valuable" £8 million SERC was to have received. "Without this money we would have had to cut the number of the best (alpha) grant applications we could support to just 60 per cent of the applications", he said at noon last Tuesday, "but now we can raise it to 70 per cent". MRC equally was hoping to raise the same figure from the "disaster" of 53 per cent this year to "somewhere in the 60s".

Sir David Phillips has meanwhile been roundly condemning the almost entirely separate way in which the different departmental budgets are arrived at — so that the science budget of Department of Education and Science, for example, cannot benefit from savings, say, in the defence development budget (which accounts for a quarter of British research and development). The Department of Trade and Industry's research budget is also not "tradeable" with the ABRC sums. An infamous (if possibly apocryphal) case being quoted last week was the "heavy armoured vehicle" whose development in the Ministry of Defence cost hundreds of millions of pounds but whose doors would open only when the vehicle was level. The errors in the defence research and development budget alone, it was suggested, could bail out British basic and strategic research. But such balancing — which should take place in the Treasury as Britain has no overall science minister — never takes place. It is time for a change, it is being said more and more loudly in the scientific corridors of power.

Robert Walgate

Students granted a reprieve

SIR Keith Joseph's retraction of his proposal to make better-off parents contribute to their children's tuition fees means that individual parents will be up to £520 a year better off at an expense to the government of £21 million in England and Wales. To find the £11 million that the Treasury will not provide, Sir Keith has reduced by £3 million the proposed increase to the science budget, by £6 million the proposed £10 million increase in the universities' equipment grant and by £2 million the increased grants in the Professional and Industrial Commercial Updating Programme (PICKUP), adult education and the microelectronics programme.

An inquiry into the whole subject of students grants is to be set up by Sir Keith to avoid the same kind of embarrassing miscalculations as occurred last Wednesday. No date for the review has been set, nor is there any guarantee that it will be an independent inquiry, but it will include the controversial suggestion that loans be made to students for their tuition and living expenses. The National Union of Students (NUS) strongly opposes this idea, believing that it discriminates against students from poorer backgrounds and those studying for a degree that does not relate directly to a business or profession. The organization does, however, welcome the suggestion of a review; at present 46 per cent of students do not receive the full extent of the deemed parental contribution, and now that the

minimum award of £205 a year has been abolished, many students will receive nothing unless their parents support their decision to gain a degree.

The Committee of Vice Chancellors and Principals (CVCP) hopes to be included in any review of the grant process. Tired of the government ignoring detailed annual reports on how much students need to live on, CVCP intends to set up its own inquiry to advise the government in any case. As the last review on the subject was in 1962, the need for a new inquiry is pressing. NUS would like to see the whole concept of parental contributions abolished; under the present system, a student is not considered independent until the age of 25.

Just how the cuts in the science budget increases will be implemented is not yet clear. The Department of Education and Science is leaving the details to the boards concerned. This must be of small comfort to the University Grants Committee which meets tomorrow; originally it was to discuss how to distribute the extra £10 million awarded to it for equipment by Sir Keith, but now its task is to divide up the much smaller cake of £4 million extra. Instead of being able to grant equipment money to three "broad areas" of science, it will now have to decide on one such area. There is a general condemnation of "robbing Peter to pay Paul", where either the students or the research programme in Britain will be the loser.

Maxine Clarke

AIDS screening

False test results raise doubts

Washington

THE crash effort by the Public Health Service, announced in April, to develop a blood test for AIDS (acquired immune deficiency syndrome) has run into difficulties. A pilot trial carried out by the Food and Drug Administration (FDA) within the past month indicates that the test kits being developed by five different contractors show wide variations in their sensitivity and selectivity, and may be subject to significant false positive and false negative rates.

The test kits all use the ELISA (enzyme-linked immunosorbent assay) method for detecting the presence in the blood of the antibody to the AIDS virus, HTLV-III. In the recent pilot trial, the five companies were each supplied with approximately 3,000 samples from plasma donations and 3,000 from whole blood donations. According to researchers who have seen results from the plasma samples, the five companies obtained positive readings on a widely varying number of samples, from a low of 5 to a high of about 100 out of 3,000. If the 3,000 samples were representative of the general population, some 20 to 30 positives would have been expected.

Any interpretation of these findings, however, is complicated by the fact that the five companies each received a different set of 6,000 samples. Each company then ran its own confirmatory tests on the positives it obtained on the ELISA tests. These confirmatory tests, using the so-called Western blot technique (an independent and more selective test), suggest a false positive rate of one out of every ten that were scored positive by the ELISA test.

According to Lowell Harmison of the Office of the Assistant Secretary for Health, duplicate samples were not initially sent to each of the companies because one object of the trial was to assess the prevalence of AIDS in the population, a goal that required a larger number of independent samples. And he said that controls on the results will be obtained by having all positive samples retested by all five companies, as well as by an FDA laboratory. He added that it is misleading to derive a false positive rate from the comparison of the ELISA tests with the Western blot tests because a consensus has only this week emerged on the definition of a positive on the Western blot.

John Petricciani, head of FDA's division of blood and blood products, which was in charge of the pilot trial, acknowledged that the trial shows that "some companies have more work to do than others in refining their tests", but that the primary purpose of the trial was to provide "an early look" at the performance of the test kits. Despite the setbacks, the Public Health Service is still predicting that the first tests will receive FDA approval and be on the market in the first quarter of 1985.

Some 14 million units of blood are collected each year in the United States from about 3-4 million individual donors. A false positive rate of 1 in 10 positives, combined with an overall incidence of 1 per cent, implies that as many as 4,000 donors would be incorrectly identified as having AIDS antibody each year.

Dr Peter Page, director of the American Red Cross blood services for the north-eastern United States, said he was concerned that the tests may be rushed to market before this false-positive problem is corrected. He said that any test goes through a development stage, in which problems are identified and resolved, but that "we're being rushed so much by Margaret Heckler [Secretary of Health and Human Services] that we don't have time

to resolve them" in the case of the AIDS test. Page noted that appeals to members of high-risk groups voluntarily to abstain from giving blood have already significantly improved the safety of the blood supply; there is no compelling case to rush ahead with blood tests that would "add a relatively small increment of safety", he said.

Although some improvement may come with better calibration of the ELISA tests, the test may prove to be essentially problematic. The test uses virus antigen to detect antibody; preparation of pure antigen is complicated by the presence of the cells used to grow the virus and the tendency of the fragile antigen-bearing coat of the virus to be lost in processing. In addition, some carriers of AIDS virus may show no antibody response whatsoever. A more advanced approach to detection might be a test that looks for the virus antigen directly in the blood.

Stephen Budiansky

British AIDS

Paper prophylaxis backfires

GOVERNMENT assurances about steps to combat the spread of AIDS (acquired immune deficiency syndrome) in Britain seem to have done as much to worry as to reassure people. One part of the British government's plan is the new Blood Products Laboratory at Elstree, north of London, opened two weeks ago by Mr John Patten, Minister of Health, which can, among other things, produce factor VIII, the blood-clotting factor used in the treatment of haemophilia.

But David Watters of the Haemophilia Society is not convinced that Elstree can meet the demand. "What the United Kingdom needs to do is invest in plasma research", he said. The total amount of plasma donated in England and Wales accounts for only 30 per cent of the annual requirement. On the same day that Mr Patten spoke of Elstree being self-sufficient, he announced further plans for heat-treated factor VIII. A spokesman for the Department of Health said "at least the means to become self-sufficient will be there". But, as David Watters points out, "it's no good having all these factories and nothing to process through them".

Meanwhile, according to Mr Patten, the war against AIDS is being waged on four fronts. The Medical Research Council (MRC) is supporting research projects at St Mary's Hospital, London, on the immunology of AIDS, at the Institute for Cancer Research on retroviruses associated with AIDS, and at the Middlesex Hospital. The MRC working party on AIDS, under the chairmanship of Dr David Tyrrell of the Common Cold Unit, was set up in the summer of 1983 and co-ordinates these projects. One of its members, Dr Richard Tedder of the department of virology at Middlesex Hospital, told a conference organized by the Terence Higgins Trust a

week ago that monogamy is the only way to restrict increasing incidence of the disease. There is some evidence that the homosexual community is taking this advice to heart, according to a survey published in the *British Medical Journal* in October.

The Communicable Disease Surveillance Centre at Colindale in London constitutes the government's second front, collating all the available information on the disease and its incidence.

In the area of public education, events move sluggishly. The Department of Health is now reprinting a leaflet circulated by the National Blood Transfusion Service (NBTS) because an earlier version discouraged from giving blood only homosexual men prepared to describe themselves as promiscuous; the new version discourages homosexual and bisexual men altogether. People who have taken drugs, visited Haiti or had any connections with central Africa (where AIDS may have originated) are also excluded.

Something similar has happened to the Health Education Council's pamphlet, *Some facts about AIDS*, due out in January 1985. Leaflets are, of course, only as effective as their circulation. A receptionist at a NBTS centre was recently asked why no such warning to prospective blood donors was on display. "We did have them out", she said, "but they frightened our customers away". Dr Harold Gunson of NBTS admits to some haphazard distribution in the past, but promises "efforts will be made to ensure that all donors attending the clinic receive a copy".

Many of the problems faced by blood banks would be solved if a reliable screening test were available. The Department of Health claims that such a test has been developed by Dr Tedder's team at the Middlesex Hospital.

Hugh Barnes

Polish universities

Setback for liberalization

Warsaw

WARSAW University's new rector, Dr Grzegorz Bialkowski, a physicist and non-Party "moderate", was elected last week in place of the "liberal" Dr Klemens Szaniawski, whose election, last May, was vetoed by the Minister of Science, Higher Education and Technology, Dr Benon Miskiewicz. The elections took place under democratic procedures laid down during the Solidarity era, procedures which, it is feared, will shortly be modified to increase ministerial control.

The liberalization of higher education under Solidarity was embodied in a new Higher Education bill which was not in the event made law until May 1982, five months after the declaration of martial law. By that time a number of amendments had been introduced, strengthening the power of the minister to overrule university decisions. Last May, Dr Miskiewicz used these powers to veto the elections of the rectors of four institutions (Warsaw, Wroclaw and Poznan Universities and the Higher School of Fine Arts). When Warsaw University tried to resist, the minister simply suspended the university's electoral college for six months and extended the term of the retiring rector, Dr Kazimierz Dobrowolski.

Hopes that such ministerial interventions would be kept to a minimum were raised at the beginning of the current academic year, when it was announced that the suspension of the electoral council would be lifted and new elections held as soon as the six months had expired. This optimism, however, was considerably reduced a few weeks later when documents began to circulate in Warsaw and other university towns setting out what purported to be proposed changes to the 1982 Higher Education on Act, which would considerably curtail university autonomy. The alleged changes included: reduction of the election of rectors to a choice between two candidates nominated by the minister; strengthening of the powers of the minister to suspend lecturers and/or collegiate bodies if he deems that they have been acting "contrary to the interests of society"; virtual abolition of participation by students and junior staff in decision-making within the university; reorganization of the *samorząd*, the non-aligned students' "self-government body", on the basis of existing student and youth organizations, all of which are Party-orientated.

Such allegations, Miskiewicz announced, were entirely false and "provocative". Nevertheless, in a meeting with the Main Council for Science and Higher Education on 15 November, he outlined his own proposals for amending the 1982 act, which according to participants were simply the proposals of the "provocative" document

in somewhat less specific form. The Main Council, which consists of one delegate from every state institution of higher education in Poland, strongly opposed the changes, noting that the 1982 act is, for "a significant fraction of the university milieu", the symbol of academic autonomy, and urging that to change a law barely two years after it came into force can only lead to a sense of doubt and instability. Even the least disapproving of the Main Council members are said to take the view that the law should be allowed to stand at least for another three years (a rector's term of office) and that any amendments should then be based on consultations and open discussions.

Students, of course, are particularly perturbed by the proposed reform of the *samorząd*, which would effectively

transfer into Party hands both such consultations on university policy as would still be left to the students after the reforms and also a whole range of welfare and social matters. Although the 1982 act forbids the formation of *samorząd* structures above the level of the individual university, representatives of the *samorządy* of all Polish universities and high schools met recently in Poznan to discuss the threatened changes. This technically illegal meeting was allowed to take place, although all participants had their identity documents checked by security police patrols in the vicinity of the assembly point. Although the content of the meeting remains confidential, it is understood that the *samorządy* as, for the moment, legally-constituted organizations, hope to be able to voice their concern through the peaceful consultative procedures provided by the 1982 act, and to restrain the more radical student elements from such proscribed activities as strikes and demonstrations.

Vera Rich

European cooperation

All-optical computer in sight

Brussels

THE European all-optical computer is just around the corner or, more precisely, less than a year away. Following a major development in optical logic gates, demonstrated in London in November and in Brussels last week, the prototype of the all-optical computer is to be built at Edinburgh's Heriot-Watt University by a team headed by Professor Desmond Smith.

The operation is part of two-year project in the field of all-optical information processing based on the concept of optical bistability. Eight research teams have collaborated in the European Joint Optical Bistability Project, which will cost 1.8 million European Currency Units (ECU) — about £1 million. They work at the Université Libre de Bruxelles (Belgium); University College Dublin (Ireland); the Centre National de la Recherche Scientifique in Strasbourg (France); the Universities of Milan, Pisa and Florence (Italy); the University of Frankfurt, the Fraunhofer Institute of Instrumental Physics in Freiburg, the Max Planck Institute of Quantum Optics in Munich and the Technical University of Berlin (West Germany); and the Royal Signals and Radar Establishment, Malvern (United Kingdom). The teams were brought together by the Community's Committee for the European Development of Science and Technology (CODEST) which promotes cooperation between research institutes and scientists.

In *Nature* last January (307, 315; 1984), Professor Smith described new developments in optical bistability and the coupling of optical logical gates, in the infrared spectrum at cryogenic temperatures, which can switch with milliwatt laser beam power

and give sufficient output to switch further interconnected elements.

The latest breakthrough involves the coupling of optical gates at visible wavelengths using the semiconductor zinc selenide, one of the many new materials tested, at room temperature. The immediate advantage of the process is that it greatly facilitates construction and could revolutionize computer design. It could also be used in arrays and displays.

The technology is similar to that used in interference filter technology: the thermally-evaporated thin films are cheap and easy to produce and, being uniformly thick, can be used, unlike to earlier technology, for large array systems and two-dimensional devices.

The disadvantage is that the use of thermal effects slows down the process but, says Professor Smith, the possibility of having many elements in parallel will increase overall speed. Limitations, he says, are now only likely to be in the order of sub-microseconds or even tens of nanoseconds.

There will be benefits in processing information from radar arrays, sonar devices and video signals, he says. Eventually it might be possible to make serial parallel conversions and to store signals in optics for longer periods of time, in fact to improve all computing processes that benefit from parallelism (for example, pattern recognition input devices reading information in parallel). Optically bistable devices could also be a better means of translating fibre optics signals.

According to Professor Smith, it will now be possible "to begin to construct digital optical circuits more complicated than demonstrated with two elements and go on from there to create optical computer architecture".

Anna Lubinska

Fast reactors

Britain falling behind?

BRITAIN'S nuclear engineers were making a strong plea last week for a British commitment to the development of fast reactors — little over a year after the government announced there was no need for haste. Nothing has changed, of course, in the engineers' perception of need: they say Britain needs the reactor as part of an arsenal of energy sources for the next century. What has changed, however, is the engineers' perception of politics. Since January, Britain has been signatory to a five-nation European umbrella agreement on fast reactor research and development, and there are growing fears that Britain will become second fiddle to France.

The plea last week came from Dr Tom Marsham of the United Kingdom Atomic Energy Authority (UKAEA), at a dinner for members of the British Nuclear Energy Society and British Nuclear Forum, that "we need to follow present plans enthusiastically and avoid further delay".

Whispers at the tables, however, indicated that the future may be with TOR, a new fast-fuel reprocessing plant under construction at Marcoule in France. Until TOR, Britain was generally thought to have the lead in efficient fast-fuel cycle management with a plant at Dounreay.

The preference for TOR gains significance in the context of an emerging consensus that Europe should develop just one major fast-fuel reprocessing plant to cope with all the fuel from the three commercial demonstration fast reactors (CDFRs) that are ultimately expected to be built in Europe (in France, West Germany and the United Kingdom). Since it is expected that Britain will have the last of the three CDFRs, it would be convenient, at least to British nuclear engineers if Britain were to win the reprocessing plant. If nothing else, it would help UKAEA to attract the engineers it needs.

Meanwhile, however, all is not going entirely smoothly on the European mainland. Electricité de France is not happy with the £2,000 million costs of Superphénix, the 1200 MW French CDFR due to start producing electricity at the end of next year, and sees no immediate prospect of further investment in that area.

And in West Germany, the increasing political power of the Greens, the environmental grouping which has now allied with the Social Democratic Party, may prevent any further fast reactor developments (such as the proposed CDFR, SNR 2).

Robert Walgate

Space station

Budget cuts spell delay

Washington

SPENDING cuts proposed by President Reagan in his effort to limit the federal budget deficit without increasing taxes are likely to delay completion of the space station now under development by the National Aeronautics and Space Administration (NASA). NASA services are among the targets listed in a budget plan presented to the cabinet last week, and NASA officials hold out little hope that the agency can escape unscathed at a time when, for example, one cost-reduction measure under consideration is a 5 per cent pay cut for all civilian employees of the federal government — which would, incidentally, also include NASA scientists.

Last year, NASA was told to expect 1 per cent real growth per year between 1986 and 1989 in order to allow development of the space station. Although NASA has traditionally been relatively immune from budget restrictions, the scale of the savings now being sought — \$34,000 million in fiscal year 1986 — means that few agencies can be permitted unnecessary increases. A decision to abandon the 1 per cent annual growth target would leave NASA administrator James Beggs with some hard decisions.

Although President Reagan is publicly committed to the space station project, the project is still in a very early stage of

development and could be postponed without incurring huge additional costs. A recent unfavourable report on the space station from Congress's Office of Technology Assessment might increase pressure for the project to be put back. One difficulty, however, is that when the President announced his plan for a permanent manned space station, in January 1984, he directed NASA to complete development "within a decade". A decision to abandon that target would therefore have to be approved by the President. Initial development costs for the space station — without instrumentation — are put at \$8,000 million.

Other contenders for programme delays are scientific projects for which funds have not yet been approved. Possibilities include TOPEX (Topographic Experiment), an ocean surveying satellite, and the advanced X-ray astrophysics facility planned for the 1990s. The Mars geoscience/climatology orbiter has also been suggested.

The Hubble space telescope, now nearing completion in time for a 1986 launch, is unlikely to be affected by any budget reductions, say NASA officials. Although the project has been subject to many delays due to technical difficulties and budget stringencies, there are now no outstanding obstacles.

Tim Beardsley

Nature Conservancy

Chief geologist resigns

THE British Nature Conservancy Council (NCC) was both criticized and rewarded last week. Dr George Black, NCC's chief geologist for the past 24 years, resigned because he considered recent developments in NCC policy alarming in their "uncompromising militancy". The government, however, has made it clear that it will support a private member's bill, tabled by David Clare, the opposition spokesman on the environment. The bill seeks to arm NCC against landowners who exploit of an anomaly in the Wildlife and Countryside Act of 1981 and destroy their land during the three months before its registration as a special, protected site. If the bill becomes law NCC will be further empowered to block any work on a prospective site that might be harmful.

In accordance with the terms of the Wildlife and Countryside Act, NCC is resurveying the 4,000 Sites of Special Scientific Interest (SSSIs) and "renotify-ing" owners and users of their obligations under the act, a costly and labour-intensive process. Earlier this year it was claimed that NCC has neither the money nor the manpower to carry out these duties but an increase of 50 per cent in its grant-in-aid budget of £7 million has recently been announced.

Dr Black's resignation, however, has been induced by the council's declared intentions on the selection of additional sites, and its strategy for the future, as published in *Nature Conservation in Great Britain* last June. Dr Black regards conservation as an applied science. "Previously, NCC saw its role as the government's scientific adviser on the natural environment. The report is beginning to introduce other concepts, such as conservation as a cultural matter." According to Dr Black, this marks a departure from its long-established tradition. In a farewell letter to his staff, Dr Black explained that differences grew out of NCC's commitment to the conservation of "a flood of scientifically sub-standard SSSIs for cultural, recreational, inspirational and spiritual reasons". Despite his anxiety to correct press reports that have presented the disagreement rather melodramatically, in terms of feuds and bad blood, Dr Black is resolute. "I've come to the conclusion, as a scientist, that I want to work for a purely scientific organization."

As far as departures from tradition go, some council members feel that NCC's earlier practices were ineffective and in need of change. In the past few years, NCC has been moving not towards confrontation and *diktat*, but very approximately into line with the opinions of the general public who finance it.

Hugh Barnes

Soviet space programme

Halley mission on course

THE era of exploration of Halley's comet is at last beginning to unfold. In two days' time, the first of a clutch of satellites due to pass by Halley sometime in March 1986 should be launched. VEGA, or "Venus-Halley", a Soviet-French collaboration which will drop probes into the atmosphere of Venus on the way to Halley, should get away this Saturday.

One French participant described VEGA recently as "a fantastic mission, really gorgeous". Soviet space technology is sometimes rough-and-ready in comparison with that of the United States, but VEGA has some beautiful equipment, according to Jean-Loup Berthod of the Verrières-le-Buisson space laboratory of the Centre National de la Recherche Scientifique.

For instance, VEGA will carry the first planetary mission camera equipped with an advanced charge-coupled-device (CCD) imaging surface, says Berthod. CCDs are "much more flexible" and linear than the standard "videcon" detection system, and "the future for space exploration is CCD" says Berthod. The US Galileo trip to the outer planets will use CCD imaging. But VEGA will be first, using a camera built by the Soviet Union, Hungary and France and with a Soviet CCD. French laboratories have made calibrations of the device and "at the present time it seems to be good".

There will be two VEGAs with identical payloads, the second to be launched a few days after the first, and each should arrive at Venus in June next year. Each VEGA will then divide into two parts, one to fly on to Halley and the other to descend onto Venus ("the Soviet's pet planet", as Berthod describes it).

Each VEGA descent probe should eject a small balloon carrying instruments to measure local pressure and temperature, and a small radio transmitter which will be followed on Earth for some days by very long baseline radio interferometry (VLBI) with the US Deep Space Network and the French and Soviet networks. This will enable the first measurement of wind speeds and directions in the venusian upper atmosphere.

The French interest has been mostly to measure a sulphur dioxide profile through the atmosphere, to learn if it may be concerned with volcanic activity. Pioneer V indicated a tenfold decrease in upper atmosphere concentrations of the gas, suggesting that the high levels detected earlier may have been transitory.

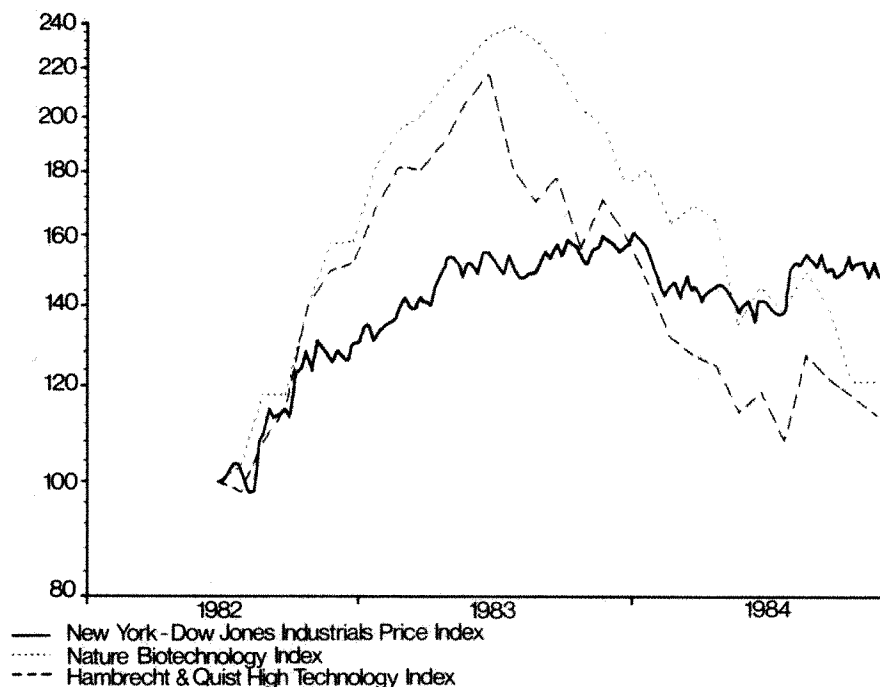
As for Halley, the first VEGA probe should reach the comet on 8 March 1986, five days before the European mission Giotto. VEGA-1 is due to pass at 10,000 km from the cometary nucleus, following the nucleus with the CCD camera on a tracking platform. VEGA information will be relayed to the European Space Agency

to help it to make the last targeting adjustment to Giotto, which is due to go much closer — within 500 km on the sunlit side and into the dust cloud. VEGA-2 will pass at 5,000 km, but it has not been decided yet whether it will go before or after Giotto (which is quite likely to be destroyed in the encounter).

"The Soviets wanted survival, and so had to go outside the large dust cloud", says Berthod. And they also wanted good pictures of the nucleus. Hence the CCD camera, and hence also the distance of approach. At a fly-by velocity of some 70 km per second, the rate at which the camera could be turned to keep track of the nucleus fixed a fly-by distance of a few thousand kilometres. Giotto will also attempt to track the nucleus, but by turning a lighter mirror system.

Robert Walgate

Comparison of biotechnology stock indexes



A comparison of the *Nature* index of biotechnology stocks (which started at 100 on 25 June 1982) with the Dow Jones and Hambrecht & Quist indexes to November 1984. (Source: Datastream/Peter Laing of Biotechnology Investments Limited.) □

Nature index of biotechnology stocks

12-Month high	12-Month low	Company	Close previous month	Close 30 November	Change
14	5 3/4	Biogen (Switzerland)	7	5 3/4	-1 1/4
2	1	Bio-Logicals (Canada)	1 5/16	1 3/8	+1/16
14 3/8	4 1/2	Bio-Response (USA)	6	4 1/2	-1 1/2
14 3/8	8 3/4	Cetus (USA)	10 1/4	8 7/8	-1 3/8
10 3/8	4 1/4	Collaborative Research (USA)	4 1/2	5 1/4	+ 3/4
19 7/8	11 1/2	Damon (USA)	12 1/8	11 3/4	- 3/8
26 1/4	11 3/4	Enzo-Biochem (USA)	18 1/4	15 1/8	-3 3/8
10 1/8	3 5/8	Flow General (USA)	4 7/8	3 5/8	-1 1/4
42 1/4	28 3/4	Genentech (USA)	28 7/8	33 1/4	+4 3/8
10 3/4	4 1/2	Genetic Systems (USA)	6 1/2	6	- 1/2
17 1/4	6 3/8	Genex (USA)	7 1/8	6 5/8	- 1/2
23	11	Hybritech (USA)	15 3/4	15 1/4	- 1/2
16 1/4	6 1/4	Molecular Genetics (USA)	7	6 1/4	- 3/4
15 1/2	8 1/4	Monoclonal Antibodies (USA)	10 1/2	9	-1 1/2
60 7/8	20 7/8	Novo Industri A/S (Denmark)	20 7/8	24 1/2	+3 5/8
22 3/4	14 1/2	Pharmacia (Sweden)	16 1/4	16 1/8	-1/8

Closing prices are for the last Friday of the month. For over-the-counter stocks, bid price is quoted; for stocks on the American and New York exchanges, the transaction price. *Nature's* weighted index of biotechnology stocks stood at 121 on 30 November, the same as a month earlier. Data from E.F. Hutton, Inc.

Teller's cold comfort

SIR — Turco *et al.*¹ have performed a valuable service by bringing to public and political attention the probability that, in addition to the "known" effects of blast and radiation, large-scale nuclear war will cause severe climatic disturbance which could increase the number of fatalities. Expressed in those simple and general terms, their claim is unassailable but, without supporting argument, it would attract neither scientific nor political interest nor would it be intellectually satisfying to the authors.

Because there was detailed supporting argument, criticism of their assumptions and model was inevitable. Predictably there has been a rapid succession of publications describing alternative analyses of the likely climatic consequences of global nuclear war, no doubt motivated in part by an understandable academic desire to arrive at the correct conclusion and by some of the other attractions of publishing in the vanguard of a topic that is attracting wide public interest. I should emphasize that this motivation is academic rather than scientific since the proponents of the various models have less chance of testing them experimentally than proponents of some of the more esoteric theories of cosmology.

Of the articles I have seen, the one that alarms me most is that of Teller². It is well argued, as one would expect, and apparently well supported factually. It is soothing in a way that the arguments from supporters of the nuclear industry often are. ("When the biological recovery formula is applied, a dose rate as high as 250 rem from worldwide fallout is built up at a rate so slow that it would not produce significant casualties". I do not wish to seem ungrateful, but this information does not make nuclear war any more appealing.)

In his introduction, Teller says: "Scientific knowledge of the after-effects of a nuclear war ... is of great importance in making political decisions". In his conclusion: "Therefore there is every reason to undertake the difficult task of arriving at more realistic predictions. ... Greater amounts of money, carefully spent on atmospheric modelling and experiments, would accelerate resolution of the basic questions regarding nuclear winter."

This, of course, is the substance of a cost-benefit analysis of nuclear war. With appropriate correction for distribution of bombs, the relation between mass of bombs exploded and the number of resulting deaths presumably resembles the Beer-Lambert spectrophotometric relation with a linear component for few bombs and a flattening of the curve for many bombs. If the "political decisions" of which Teller writes (I assume he means whether or not to bomb the Soviet Union) were to make any military sense, then the number of bombs used would have to be

small enough to ensure that they were on the linear part of the death curve. Notwithstanding Teller's point about accurate low-yield missiles, that possibility seems very remote. Moreover, the correspondence about nuclear winter is specifically based on many bombs.

If it could be demonstrated that the predictions of a nuclear winter are exaggerated, how would that knowledge be used? Would one side bomb the other forthwith, secure in the knowledge that their few survivors would not be unduly cold, at least in summer? Or conversely, if the prediction is not exaggerated, does that mean that each side should stockpile food, as Teller suggests, so that the survivors can at least have some cold, albeit radioactive, porridge? If, in more comprehensive terms, the additional research for which Teller calls should conclude that a global war would not kill everyone, but merely 90 per cent of the world's population, would that make the war acceptable? The fine details of the ostensibly rational discussion about nuclear winter are fast making that discussion the penultimate insanity.

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Bedlam at banquets

SIR — At many international congresses — phycological, entomological, and all sorts of others I've attended in recent years — the value and pleasure of the closing dinners have been considerably reduced by noise. Bands and comedians, however good, and however appropriate for youth or political rallies, are out of place at scientific meetings, particularly on the final evening which may be the last (or even the only) occasion for congenial and informal discussions.

Musicians and other entertainers, with their inevitable kilobel amplifiers, should not be included in the programmes on such occasions. Communication is hard enough as it is, especially for the partly deaf, or for Finns and Turks trying to discuss technical matters in English with Japanese and Chinese colleagues who have gathered, often at considerable expense, perhaps for the only occasion in their lives; gratuitous noise makes it harder. There is a place and a time for entertainers, but not at conferences which we attend principally for the business of conferring.

Organizers, please note.

RALPH A. LEWIN

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Plight of UK scientists

SIR — The plight of Indian scientists (*Nature* 8 November, p.87) should not blind us to the problems which British scientists have long faced. In the words of your leading article, India has had a government whose policy for science had open and straightforward objectives, namely that understanding the natural world would in due course be a source of economic prosperity, social improvement and the banishment of superstition. Britain, by contrast, has a government which has looked mainly to immediate industrial benefits from science, and has adopted a policy towards higher education that is resulting, whether the government realizes it or not, in the Dissolution of the Universities. Research has not only been stultified by lack of funding in other than a few favoured subjects, especially in our universities which should be the power-houses of research, but has also been subjected to policy planning which, by its very nature, cannot but direct resources and effort to the last problem instead of the next one. Desperate though the need for improved financial support is, it is even more vital to return to the tested and time-honoured Haldane principle in research.

Perhaps the international scientific community can indeed help science in India, but reciprocally science in Britain would be enormously helped if our national decision-makers were to learn from India.

PETER FELLOTT

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Peer review

SIR — Surely, if a "considerable element of chance is an inescapable component of today's review procedures" (Bryn Bridges, *Nature* 4 October, p.406), then the system should have been designed to take account of this random element. For example, the sharp cut-off point in the rank order when funds are exhausted would be blunted if there were a sliding scale of funding. Progressively less outstanding applications would receive progressively less than the optimum funds required to complete the project in a reasonable time. Applicants would then have to modify or slow down the rate of work. At least the entire project would not collapse. The peer review system was designed in the "good old days", when virtually any good application was sure of being funded. The system has failed to adapt to the realities of funding today's research^{1,2}.

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Opportunities in particle physics

from C.H. Llewellyn Smith

While a British committee is considering the question whether the United Kingdom can afford to remain a member of the European high-energy physics consortium CERN, particle physics itself is at an exciting stage. Here a British theorist describes the prospects for the coming decade.

THE spectacular advances in particle physics of the past fifteen years culminated in the discovery of the W and Z bosons, the carriers of the weak force, in experiments at CERN last year. These experiments have now produced tantalizing hints of quite unexpected new phenomena. This is therefore an exciting time for particle physics, and a good one to assess the progress that has been made and to look to the future.

How far ahead is it sensible to look? It is sometimes assumed that the cost of accelerators is growing inexorably so that, sooner or later, particle physics must grind to a halt. The facts tell a different story. In the case of CERN, for example, the cost of the LEP machine (the large electron-positron collider), which will come into operation in 1989, is about three-quarters of the cost of the previous accelerator, the SPS, commissioned in 1977. In fact, the total CERN budget, which includes the operation of existing facilities as well as capital costs, has fallen by about 20 per cent in real terms during the past decade, from a peak in 1974 during the construction of the SPS, and will remain constant during the construction of LEP. Looking beyond LEP to the end of the century, it seems that a very powerful proton collider could be built in the LEP tunnel without increasing the CERN budget, if that should prove desirable.

The past two decades

In order to understand the present state of particle physics, it is instructive to begin by recalling the situation twenty years ago. The known constituents of matter could be divided into two classes:

- **Leptons.** These are particles such as the electron, the muon and neutrinos which do not feel the strong, or nuclear, force. Four were then known, and it was clear that they were simple objects with no size, within the limits of experimental resolution.

- **Hadrons.** These are the particles such as the proton, the neutron, the pi-meson or pion, hyperons, and so on, which do feel the strong force. Hundreds were known and it was clear that they were complex objects with finite sizes.

It was known that four forces control the behaviour of these particles:

- **The gravitational force.** Gravity has been well understood classically since Newton's theory was perfected by Einstein, but even now there is no satisfactory quantum theory of gravity. The effects of gravity are quite negligible in experimental particle physics, and are important only for very large bodies, because gravity acts in-

discriminately and additively on all matter. Theoretically, however, it may be that we ignore gravity at our peril when studying the nature of force.

- **The electromagnetic force.** Electromagnetism is described by Maxwell's equations and Dirac's theory of the electron, which were successfully combined to form quantum electrodynamics, or QED, in the late 1940's. The electromagnetic force holds atoms together and controls the behaviour of matter in bulk.

- **The strong force.** This is responsible for holding the nucleus together; its origin was obscure twenty years ago.

- **The weak force.** This is responsible for β -decay and other rare processes. No mathematically consistent theory of this force was known.

As can be surmised from this brief summary, the immediate goals of particle physics were to determine (1) the nature of hadrons and the relationship between them, (2) the nature of the strong force, and (3) the nature of the weak force. Two decades later, it can be argued that all these goals have been achieved.

As the spectrum of hadrons was slowly established by many meticulous experiments, patterns began to emerge and it was shown that they could be explained by assuming that the hundreds of hadrons are composed of a small number of more fundamental entities, called quarks, put together in the different ways allowed by quantum mechanics. Experiments using high-energy electrons (at SLAC in California) and neutrinos (at CERN) made it possible, in effect, to X-ray the proton, revealing that it does indeed contain objects with exactly the properties previously postulated for quarks. The unexpectedly large probability for backward scattering found in these experiments revealed that quarks, like leptons, are point-like within the resolution, just as the observation of large back-scattering of α -particles by gold revealed to Rutherford that the nucleus is an extremely small part of the atom.

Once it was known that hadrons are composed of quarks, it became possible to construct a theory of the strong force. This force acts between quarks, which it holds together to form hadrons, generating nuclear binding as a secondary effect. The data led to a theory known as quantum chromodynamics or QCD.

According to relativistic quantum mechanics, all forces are generated by the "exchange" of particles. An analogy is provided by two skaters exchanging a ball while gliding on a frozen lake, who would

be forced apart by the impulses generated when they catch and throw the ball. (This analogy fails to explain how forces of attraction are generated, although the exchange of boomerangs might be invoked!) In QED, for example, the electromagnetic force is generated by the exchange of photons between electrically charged particles. Weak processes are generated by the exchange of the heavy W and Z particles. The interquark force is generated by the exchange of particles called "gluons". In QCD a quantity known as "colour" plays a role analogous to that of electric charge in electromagnetism (hence the name chromodynamics). Both quarks and gluons carry colour charge, so gluon-exchange generates a gluon-gluon force as well as gluon-quark and quark-quark forces. It seems that these forces are so strong that quarks and gluons cannot exist as individual particles, but always bind together in combinations with zero colour charge to form hadrons.

It is much harder to wrest quantitative results from QCD than from QED, because of the strength and nonlinear nature of the interactions, but various qualitative results can be derived and some quantitative predictions have been made which have been borne out by experiment. One example is the prediction that, in certain circumstances, quarks should radiate energetic gluons which would give rise to characteristic collimated jets of particles. Such jets were discovered in experiments at the electron-positron collider PETRA in Hamburg, which revealed the existence and nature of the gluon.

Not long before the development of QCD, 't Hooft demonstrated the consistency of a theory of the weak force previously proposed by Glashow, Weinberg and Salam. Consistency requires this theory to be based on a principle known as local gauge invariance, which also underlies QED, QCD and Einstein's theory of gravity. In the case of gravity, local gauge invariance means that the form of the laws does not depend on the choice of the space-time coordinate system chosen to label events, and it actually allows different coordinate systems to be used at different places. In the other cases, local gauge invariance allows certain "internal" coordinate systems to be chosen differently at different places. In QCD, for example, each quark has one of three possible colour charges: the forces depend on the relative values of these charges, but not on which colour charge is assigned to a particular quark, and the assignment of "colour

coordinates" can be different at different places. The discovery that all the known forces are based on the same principle, which implies that they have a similar mathematical structure, opens up the exciting prospect of finding a theory that unifies all forces, in the sense that Maxwell's equations unify electric and magnetic forces.

The new theory of the weak force necessarily involves a partial unification with electromagnetism. In addition to the electrically charged W , whose exchange generates all the weak processes known before 1973, it requires the existence of an electrically neutral "heavy photon", called Z , and predicted a new class of weak processes that would be generated by Z -exchange. The discovery at CERN in 1973 of some of these processes, known as "neutral current neutrino interactions", was therefore a vital first step in establishing the electroweak theory.

The effects of a force are proportional to the strengths of the charges involved and also depend on its range. The analogy of the skaters passing a ball back and forth suggests that the heavier the carrier, the shorter the range over which it can be exchanged. In fact, the range of a force varies inversely with the total energy carried by the exchanged particle, including the energy (mc^2) stored in the mass. Because the mass of the photon is very small, certainly less than 10^{-50} kg and very probably zero, it can carry a negligibly small amount of energy. Consequently, electromagnetic forces can act over very large distances, as anyone who has used a compass knows. The basic idea of the unified electroweak theory is that the intrinsic strengths of weak and electromagnetic charges are similar, weak effects being relatively very feeble because the W and Z are very heavy and therefore act only over very short distances. If weak charges are indeed related to electromagnetic charges as in the unified theory, the observed weak effects require the W and Z to be about 100 times as heavy as the proton. Until last year, the data were consistent with any masses from 20 times that of the proton to infinity. The discovery of the W and Z with the predicted masses was therefore a major step forward as it provided the first direct evidence for electroweak unification.

The outstanding questions

The description of the structure of matter in terms of a relatively small number of quarks and leptons, whose interactions are controlled by QCD, the unified electroweak theory and by gravity, has come to be known as the "standard model". Although this model successfully describes an enormous body of data, it was recognized from the outset that it is incomplete. It simply does not explain enough. Too many of its ingredients are added in amounts adjusted to fit the data (although, in fairness to the model, it

The six leptons

Three electrically charged leptons are known: the electron (e), the μ (discovered in cosmic rays in the 1940s) and the τ (discovered at SLAC in California in 1975). Each is linked by the weak force to its own much lighter (possibly massless) neutral lepton or neutrino (ν) — so W exchange transmutes e into ν_e : $e \rightarrow (W)\nu_e$ and so on. As far as is known, the properties of the three "lepton doublets"

(e, ν_e) (μ, ν_μ) (τ, ν_τ)

are identical except that their masses differ. Their masses in GeV, in which unit the mass of the proton is 0.938, are

e : 5.1×10^{-4}	ν_e : less than 5×10^{-8}
μ : 0.106	ν_μ : less than 5×10^{-4}
τ : 1.78	ν_τ : less than 0.16

The six quarks

Six quarks are known. The up (u) and down (d) quarks are needed to make neutrons and protons: they move inside hadrons with effective masses of about 0.33 GeV. The strange (s) quark, with mass about 0.5 GeV, is needed to make the "strange" particles discovered in cosmic rays in the 1940s. The charmed quark (c), with mass 1.5 GeV, is needed to make the J/ψ particle, discovered at BNL on Long Island and at SLAC in 1974, and charmed particles found at SLAC in 1976. The bottom (b) quark, with mass of 4.8 GeV, is needed to make the Υ found at Fermilab near Chicago in 1977 and "bottom" particles found at Cornell in 1983. Finally, the top (t) quark is needed to make the standard model work, given the existence of the b and of six leptons. The 'UAI' experiment at the CERN proton-antiproton collider has recently discovered an exotic class of events which can be consistently interpreted as evidence for the t with a mass of about 40 GeV. All the quarks seem to respond identically to the strong force. The weak force links pairs of quarks, as it does leptons, thus

(d, u) (s, c) (b, t)

However, "off-diagonal" weak transmutations such as $s \leftrightarrow u$ and $b \leftrightarrow c$ occasionally occur: the standard model can accommodate but does not explain the existence of these transmutations. Likewise it can accommodate but not explain the observed numbers and masses of quarks and leptons. $\square$

should be acknowledged that most of these ingredients are quantities such as the fine structure constant and the ratio of the electron and proton masses which have hitherto been regarded as given fundamental constants). If the standard model is incomplete, its predictions have only a limited domain of validity, just as the predictions of Newton's theory of gravity, which is an approximation to Einstein's more complete theory, are not valid in all circumstances. Experimental particle physicists are therefore searching for phenomena that the standard model cannot explain, while theorists are striving to construct more complete models and predict what these phenomena might be.

Three major questions are raised, or not properly answered, by the standard model:

● **What determines the number of quarks and leptons?** At present six quarks and six leptons are known (see box). As far as we can tell, the present Universe would continue to function in much the same way

with just two of each, although six or more are needed in order to explain a phenomenon called CP violation which, although very rare today, is believed to have played an essential role just after the big bang in generating the excess of matter over antimatter now observed.

The numbers and spectra of quarks and leptons are essentially arbitrary in the standard model, and attempts to find more complete theories which explain them have, so far, been woefully unsuccessful. There are many other questions raised by quarks and leptons. Are their properties connected, or is it just coincidence that, for example, six of each of them are known, or that the electric charge of the electron is an integral multiple of the charges of quarks to better than one part in 10^{20} ? Will they turn out to be composite, which might account for their number and explain these parallels? History suggests the answer yes, but there is no hint of compositeness in their properties, whereas the composite nature of neutrons and protons was suggested rather directly by their sizes, anomalous magnetic moments and the existence of excited states.

● **Can we further unify the forces?** This is an obvious aspiration, given the success of the unified electro-weak theory and the discovery that all forces have a similar mathematical structure. A first step would be to unify completely the electromagnetic and weak forces, which are actually mixtures of two independent fundamental forces according to the standard model. The fact that the charges associated with the two forces turn out to have similar strengths certainly suggests that they are not in fact independent, but are linked in a more complete theory.

Complete electroweak unification is actually quite hard to achieve except in the much more ambitious framework of "grand unified theories" (GUTs), which not only fully unify the electromagnetic and weak forces but also combine them with the apparently very different strong force. GUTs depend on the fact that "charges" (strong and electroweak) are distance-dependent because of "vacuum polarization" due to the clouds of "virtual" particles which surround any charge. This is analogous to the screening effect of polarization in a dielectric medium, which causes the charge of a test particle to be effectively reduced (by the inverse of the dielectric constant) at distances greater than the molecular spacing. In QCD, there is anti-screening and the effective strong charge of a quark decreases as it is approached. It is therefore conceivable that at very short distances, inside the effects of all screening, the fundamental strong and electroweak charges are the same. Extrapolating their known values to very short distances, it seems that the strong charge and the charges associated with the two parts of the electroweak force do indeed converge. Conversely, the GUT hypothesis leads to a successful

post-diction for the relative strengths of the two parts of the electroweak force. From the distance at which the charges appear to converge, it can be inferred that the hypothetical particles, which are supposed to generate the screening masking the connection between strong and electroweak forces, must have masses about 10^{14} times the mass of the proton. These particles may produce dramatic effects which will be considered later.

In the absence of experimental evidence for their validity, GUTs are highly speculative, but at least they seem to be consistent. This is more than can be said so far of attempts to achieve the ultimate goal of synthesizing gravity with the other forces.

● **What is the origin of the masses of particles?** This question is brought into prominence by the success of the electroweak theory because the symmetries of the equations seem to imply that electromagnetic and weak forces are identical in range as well as strength, in which case the W and Z would be mass-less, like the photon. They also seem to imply that all quarks and leptons are mass-less, contrary to the facts. Nevertheless, the equations may describe the real world because they may have solutions in which the symmetries are hidden. Analogously, in a ferromagnet the atomic magnetic moments all line up in one particular direction chosen at random, thus hiding the rotational invariance of the laws of nature. In the standard electroweak theory, an added ingredient called the Higgs field causes the equilibrium state of the theory to be one in which the unobserved symmetries are hidden, thereby allowing the W and Z, and the quarks and leptons, to acquire masses. This theory can be made to fit the facts, but it appears to be very contrived. Is it only an approximation to a more complete theory, or may some quite different mechanism hide symmetries and thereby generate masses?

All the known ways of generating masses for the W and Z require the existence of new particles or new phenomena that may appear at energies which will be explored in forthcoming experiments — typically around 100 GeV (close to the rest energy, mc^2 , of the W and Z themselves) and in all cases below 1,000 GeV. (It is convenient henceforth to use the GeV as a unit of energy and mass; the rest energy or mass of the proton is 0.94 GeV.) In the standard model, there is just one new particle, the "Higgs boson" still to be found, in which case there could then be a "desert", devoid of new phenomena, extending to the grand unified scale of 10^{14} GeV or beyond. However, there are compelling arguments to expect that, rather than a desert, a fertile region rich in new phenomena awaits exploration.

Accelerators and colliders

The main tool of particle physics is the accelerator. High energy particles are needed both because they have the small quantum

mechanical wavelengths required to resolve the structure of matter on a small scale (the principle of the electron microscope) and also to create heavy particles such as the W and Z, using $mc^2 = E$. When a beam of particles strikes a stationary target, momentum conservation requires the debris to be thrown forward. The kinetic energy associated with this motion is wasted; it cannot be used for creating new particles or probing short distances. Indeed, because of relativity, the fraction of the incident energy which is wasted increases dramatically with increasing energy (the useful energy grows only as the square root of the beam energy). This waste can be avoided by colliding two beams of particles head-on, as in the CERN proton-antiproton collider used to discover the W and Z. To produce the same useful energy with a stationary target, a beam with energy 300 times the combined energy of the proton and antiproton beams would have been required.

Because of this effect, most future experiments will involve colliders. The price to be paid is that a second beam provides a much less dense target than a stationary piece of matter, and thus a much lower collision rate. Furthermore, only stable charged particles (protons (p), antiprotons ($\bar{p}$), electrons (e) and antielectrons or positrons ($\bar{e}$)) can be stored and collided, although this is not a serious limitation in addressing fundamental questions because all combination of lepton, quark and gluon interactions can be studied with pp , $p\bar{p}$, $e\bar{e}$ or ep colliders.

The characteristics of some of the world's front-line machines, now running or under construction, are shown in the table below. One striking feature of this table is that the proton beam energies are much higher than the electron energies. The explanation is that when charged particles are deflected by the magnets that guide them around circular accelerators, some of the energy stored in the electromagnetic field accompanying the charge carries on in a straight line, generating synchrotron radiation. This radiation may be a bonus for those who can use it as a tool, but it is an expensive problem for particle physicists. To reduce the energy lost by radiation, circular electron machines must be made very large and this proves to be the limiting cost factor. Synchrotron radiation is unimportant for protons because they are so much heavier, which is why they can be accelerated to much higher energy than electrons for a comparable cost.

Nevertheless, electron machines are able to hold their own because, in a proton machine, the energy of the proton is shared among a cluster of quarks and gluons so that an individual quark or gluon typically carries only one tenth to one fifth of the beam energy. Moreover, the interpretation of proton collisions is complicated by the fact the fraction of the energy carried by individual quarks and gluons is not fixed.

The cost of circular electron machines increases very rapidly with energy because of synchrotron radiation. Above about 150 GeV per beam, it will become cheaper to collide beams from two opposed linear accelerators, and the long-term future of $e\bar{e}$ colliders depends on whether this is practicable. A first test will be provided by the SLAC Linear Collider (SLC), which will use an existing linear accelerator to accelerate beams that will be separated and collided. Since the beams will then be thrown away, a reasonable collision rate can be achieved only by focusing them to about one micrometre in diameter. Producing and colliding such beams is a formidable challenge, comparable with that faced in producing and colliding intense bunches of antiprotons at CERN.

The experimental programme

Experiments at colliders are carried out with very large detectors which surround the collision point and are designed to measure the energy, momentum and nature of the particles produced. Every detector is a compromise, and different detectors are designed to have different strengths in their abilities to study different types of particle, to measure energy and so on. Some idea of their typical size is provided by the UA1 detector at the CERN $p\bar{p}$ collider, which is a $10\text{m} \times 10\text{m} \times 10\text{m}$ cube packed with sophisticated equipment. Such instruments are always used for several years, with each period during which the accelerator is run corresponding, in a sense, to a new experiment, because the apparatus is often modified and the way it is operated is changed in the light of previous results. For example, in each run, suitable trigger conditions must be chosen to recognize instantaneously especially interesting categories of events whose characteristics are recorded for later detailed study, because the total number of events is usually far too large for all to be recorded and analysed.

How will future experiments cast light on the grand problems of particle physics? Two caveats apply to what follows. First, inevitably, the discussion must be confined to the framework of existing speculation. Experiments in the next decade will probe the structure of matter at one-tenth of the distance scale explored so far, and it will be surprising if they do not produce surprises. Second, the discussion is concerned only with measurements bearing on really fundamental questions. Future experiments will also allow detailed studies of phenomena whose fundamental character is already known, but about which there is nevertheless still much to be learned.

The discussion will be organized on the basis of theoretical problems rather than machines, but it will become clear that different types of collider ($p\bar{p}$, $e\bar{e}$, ep) have complementary capabilities. Very crudely, $p\bar{p}$ and pp colliders reach the highest energy, but are relatively blunt exploratory instruments compared to $e\bar{e}$ and ep

machines. For example, while pp collisions yield some information about the distribution of quarks and gluons in the proton, it is crude compared to what is learned from "X-raying" the proton in ep collisions. Similarly, although the Z and probably the t quark have been discovered at a $p\bar{p}$ collider, investigation of their detailed properties must await SLC and LEP. Proton-antiproton collisions at CERN have already produced tens of Zs, but electron-positron collisions will resonate at the rest energy of the Z, which may be produced at a rate of tens, or perhaps hundreds, of thousands per year in the SLC experiment and millions per year in each of the four simultaneous experiments planned for LEP. This will allow high precision studies of the Z itself, and of its decay products, including the t quark and any as yet undiscovered particles less massive than the Z. Finally, although existing $p\bar{p}$ colliders have enough energy in principle, only LEP when upgraded to more than 85 GeV per beam will be capable of studying the production of pairs of Ws so allowing crucial tests of the electroweak theory. This is why the LEP design is optimized for eventual running at 100 GeV per beam.

Future experiments will address the "grand questions" in the following ways:

● **Particles.** New charged particles can most easily be found in $e\bar{e}$ collisions, in which an electron and positron annihilate forming an unstable quantum of electromagnetic energy which disintegrates into whatever charged particles it can. From PETRA experiments, we know that there are no charged leptons between the τ , with a mass of 1.7 GeV, and about 20 GeV. SLC will extend the search to 50 GeV, while LEP will eventually explore the region up to 100 GeV. SLC and LEP will make detailed studies of the t quark, if its mass is around 40 GeV as suggested by recent CERN data, and LEP will eventually discover any other quarks with masses less than 100 GeV.

If there are additional neutral leptons (neutrinos) with masses less than half that of Z, it will decay into them. Although such decays cannot be observed directly, they would alter the Z's half-life in a way that could be inferred from experiments at $p\bar{p}$ and $e\bar{e}$ colliders. We have already learned from the CERN $p\bar{p}$ collider that there cannot be more than about five such leptons. If any exist, they will be discovered at SLC or LEP. In addition to the obvious importance for particle physics, knowledge of the number of light neutrinos is vital for astrophysics, for it determines how fast the Universe expanded when it was a few minutes old, which in turn controls the cosmic abundance of helium. The number of neutrinos also influences the fraction of the energy of the Universe which may now be stored in neutrino background radiation, a quantity which is very sensitive to whether neutrinos actually have small masses, as is strongly suggested by GUTs. This question is now being vigorously

		Some front-line facilities	
Europe	Existing	Under construction	
CERN	SPS 450 GeV p $E_{cm} = 30$ GeV	LEP (1989) 50 GeV $e + 50$ GeV $\bar{e}$ $E_{cm} = 100$ GeV ($E_{cm} \approx 200$ GeV, 1992?)	
	SPPS 320 GeV $p + 320$ GeV $\bar{p}$ $E_{cm} = 640$ GeV		
DESY	PETRA 23 GeV $e + 23$ GeV $\bar{e}$ $E_{cm} = 46$ GeV	HERA (1990) 35 GeV $e + 900$ GeV p $E_{cm} = 350$ GeV	
USA	FNAL Tevatron 800 GeV p $E_{cm} = 40$ GeV	TeV (1986) 1,000 GeV $\bar{p} + 1,000$ GeV p $E_{cm} = 2,000$ GeV	
SLAC	PEP 15 GeV $e + 15$ GeV $\bar{e}$ $E_{cm} = 30$ GeV	SLC (1987) 50 GeV $e + 50$ GeV $\bar{e}$ $E_{cm} = 100$ GeV	

The acronyms of each laboratory are listed in the left-hand column. Data given for each machine are: completion date, in parentheses; energy of beam in GeV, type of particle [proton (p), antiproton ($\bar{p}$), electron (e), positron ($\bar{e}$)] + energy of opposing beam and type of particle in the case of colliders. E_{cm} = centre of mass energy; this is the useful energy available for creating new particles. 1 GeV is slightly more than the energy (mc^2) of a proton at rest, which is 0.94 GeV. A machine with $E_{cm} = 50$ GeV can in principle create a particle 50 times as heavy as the proton.

attacked in various relatively low energy particle physics experiments. Some astrophysicists claim that the observed abundance of helium makes it unlikely that there are more than one or two undiscovered light neutrinos. A direct measurement in Z decay, which could also find heavy neutral leptons, is obviously highly desirable.

If quarks and leptons are composite, they may have detectable structure and excited states should exist. According to one school of thought, the W and Z are also composite and share common constituents with quarks and leptons. If so, structure should show up at a distance scale set by the mass of the W that will be probed by experiments at $p\bar{p}$ colliders, at SLC, LEP and HERA, which will, in their different ways, extend the search for structure down to one-tenth of the distances studied hitherto. Enthusiasts for composite models claim that the unexplained events recently observed at the CERN $p\bar{p}$ collider may be manifestations of excited states of quarks.

● **Forces.** Further tests of the unified electroweak theory are badly needed for two reasons. First, it is still conceivable that the W and Z are composite particles, unconnected to the photon. It can be argued that, nevertheless, they might have properties similar to those predicted by the standard model if they are very tightly bound states of constituents with masses of about 1,000 GeV. In that case, however, their properties would be nonstandard to a degree that should be measurable in forthcoming experiments and there would also be heavier particles made of the same constituents. Second, if, as seems much more probable, the standard model is basically correct, it is very likely that it is part of a completely unified theory. If so, there must be heavier W and Z-like particles and the ordinary W and Z might have slightly non-standard properties.

Many new tests of the electroweak theory will become possible in the coming decade. At SLC and LEP, accurate meas-

urements of the mass of the Z and of the cross-section for the production of muon pairs will be particularly important. An upgraded LEP will also be able to study W pair production, which is sensitive to basic untested features of the electroweak theory and may serve to distinguish it from models in which the W is composite. Experiments at an upgraded LEP will also be sensitive to the effects of additional Zs with masses up to about 500 GeV. Experiments at HERA which study electron-quark scattering, and the reaction electron + quark $\rightarrow$ neutrino + quark, will allow independent high-precision tests of the electroweak theory and will be sensitive to the effects of additional charged Ws with masses up to about 500 GeV, which could not be seen at SLC and LEP.

In GUTs, some, perhaps all, of the additional Ws and Zs are supposed to have masses of the order of 10^{14} GeV. Exchange of these very heavy particles is expected to generate proton decay. Since, by definition, leptons do not feel the strong force, while quarks do, it is not surprising that in GUTs, in which there is no fundamental distinction between the strong and the electroweak forces, there is no fundamental distinction between quarks and leptons. Thus GUTs unify quarks and leptons, as well as unifying forces, and GUT forces can transmute quarks into leptons thus allowing the proton to decay.

The prediction that the proton is unstable is exciting first because it alters our view of the permanence of matter, second because it may explain the imbalance between matter and antimatter in the Universe, and, third, because it may be testable. Large detectors have been constructed to examine the stability of large quantities (hundreds or even thousands of tons) of matter in deep mines, shielded from most of the effects of cosmic radiation. These experiments are at an exciting stage, having already reached the sensitivity required to see proton and neutron decays according to some varieties

of GUTs. They have found a few events that defy simple explanation but more results, and perhaps the more sophisticated detectors now being constructed, will be needed to determine the origin of these events. These experiments are also sensitive to any powerful cosmic sources of neutrinos (they might eventually be able to detect neutrinos from supernovae) and to any new highly penetrating particles that might be present in cosmic rays, such as the magnetic monopoles whose existence is predicted by GUTs.

● **Masses.** The known solutions to the problem of why the W and Z are massive all involve new phenomena that may be discovered in forthcoming experiments. The photon, which is believed to be massless, can exist in two independent states, which is why ordinary light waves can be polarized in two independent ways. But "heavy photons" such as the W and Z can exist in three independent states. (Technically the photon has spin one: if it were massive, we could always find a frame of reference in which it was at rest and then orientate the spin in three independent ways.) The question of why the W and Z are massive while the photon is mass-less, although it is their sibling according to the unified electroweak theory, is therefore directly related to the origin and nature of their third states. There are three possibilities:

- (1) The third states of the W and Z may be fundamental particles. This is the case in the textbook formulation of the standard model, which contains fundamental entities called Higgs fields, or particles, which induce a preference for a solution in which the symmetry is hidden. The Higgs particles supply the third states for the W and Z, thus distinguishing them from the photon and enabling them to become massive. However, it turns out, inevitably, that one Higgs particle is left over as a new independent type of particle called the Higgs boson. Its mass is not predicted by the theory, but must be less than a few hundred GeV for the theory to make sense. Searches for this particle are obviously extremely important. Only $e\bar{e}$ colliders provide a reasonable chance of finding it. If its mass is less than 50 GeV, it may be found in high statistics studies of Z decay at LEP. If it is heavier, it may be produced in conjunction with a Z at an upgraded LEP.
- (2) The third states of the W and Z may be composite particles. The hypothetical constituents of these composite particles are usually referred to as "techni-quarks", and the force which is supposed to bind them together as "technicolour". It is difficult to make technicolour models which are compatible with all the data, but they have some very attractive features and must be taken seriously. If the underlying idea is correct, there should be other bound states of the constituents which can be sought at SLC and LEP, and non-standard behaviour may show up in W pair production at an upgraded LEP.
- (3) All three states of the W and Z may be

composite and unrelated to the photon, in which case the mass problem would be transferred to the constituent level. As discussed above, this possibility will be tested in future experiments.

Among these possibilities the "Higgs mechanism" for mass generation is the most conservative in the sense that it predicts the existence of only one additional undiscovered particle. However, the masses of the W and Z introduced by the Higgs mechanism are unstable, and tend to be dragged up to the largest mass scale available, presumably the 10^{19} GeV scale associated with quantum gravity. In the standard model, they can be held down at their observed values, of about 100 GeV, only by a highly artificial balancing of the parameters in the theory, which must be "tuned" to an accuracy of one part in 10^{26} . This instability can be avoided only by introducing a new hidden symmetry, called supersymmetry (SUSY). SUSY is appealing for many reasons. It unites particles with different quantum mechanical spins in common families. It provides a natural framework for unifying gravity with other forces, since when SUSY is made local, in the sense of local gauge invariance, it turns out to be part of a theory that includes Einstein's theory of gravity. Finally, according to rather general theorems, SUSY is the only type of symmetry possible for which there is not already some experimental evidence. It would seem parsimonious of nature to have exploited all symmetries but one, so we should take the possibility of an underlying supersymmetry very seriously.

According to SUSY, every particle should have a "superpartner" whose quantum mechanical spin differs by half a unit. Thus, corresponding to the known quarks and leptons which have spin one-half, there should be spinless quarks and leptons, known in the trade as squarks and sleptons, and there should be spin one-half partners of the W, the Z, the photon and the gluon, which have spin one. It turns out that, in order to avoid the instability that led us to SUSY, these superparticles must lie below 1,000 GeV in mass and, in most models, some of them have masses similar to that of the W. If this is so, there is a good chance that they will be found in the next generation of experiments. Experiments at the $p\bar{p}$ collider may discover squarks and spin one-half gluons provided they are not much heavier than 50 GeV. Enthusiasts for SUSY claim that the recent unexpected results found by these experiments may already provide evidence for superparticles. Experiments at HERA may discover some particular squarks and sleptons with masses up to about 150 GeV, the exact limit being model-dependent. SLC or LEP will find any charged superparticles, and quite probably also neutral superparticles, whose masses are less than 50 GeV. Upgraded LEP will extend the search to 100 GeV. If superparticles exist, $e\bar{e}$ experiments will provide the best way to

study their properties in detail.

Conclusions

There has been remarkable progress in particle physics in the past two decades. Our description of the observed particles has been greatly simplified by the discovery of an underlying quark substructure. Following the triumph of the gauge principle, it is perhaps not too much to claim that we now know the origin of force. This progress has been synthesized in the so-called standard model, which successfully describes a vast amount of data, but it has been recognized from the beginning that this model cannot be complete. Theorists have been searching energetically for more complete models, but there are too many options and the baton is now in the hands of our experimental colleagues.

The present experimental situation is exciting. Experiments at the CERN $p\bar{p}$ collider and proton decay experiments have recently given hints of phenomena that may lie outside the framework of the standard model.

With the Fermilab $p\bar{p}$ collider, SLC, LEP, and HERA under construction, there is a rich experimental programme ahead that will address many fundamental questions. Experiments in the next decade will extend our knowledge of the spectrum of quarks, leptons and any other particles to much higher masses. They will test the unified theories of forces. They may reveal the origin of the masses of the W and Z, which seems to be intimately linked with the origin of all masses. They will explore uncharted energy and distance scales and may reveal phenomena as yet undreamt of.

Looking beyond the machines now under construction, it is already clear that a 12,000 GeV proton collider could be built in the LEP tunnel at CERN for much the same cost as the LEP project, and energies up to 20,000 GeV may become possible; as well as pp collisions, such a machine could generate ep collisions at six or more times the HERA energy, since one beam could be collided with an electron beam in LEP. Scientists in the United States are considering a more ambitious 40,000 GeV proton Superconducting Super Collider (SSC), which would be far more expensive because it would be much larger and would require the construction of a new tunnel. Indeed, construction of the SSC would require the annual US particle physics budget to be doubled, but even this increase would only restore expenditure to the same fraction of GNP as in the mid-1960s. It is therefore possible to imagine an experimental programme stretching a quarter of a century ahead, if it is scientifically desirable. In the more distant future, novel techniques will have to be found to contain the cost of accelerators if it proves imperative to go to even higher energies. □

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Nuclear winter and carbon dioxide

The National Academy of Sciences has at last reported on the nuclear winter. It clarifies the scientific uncertainties that affect other important climatic models.

THIS week's report on nuclear winter by the US National Academy of Sciences may be late (by more than a year), but is nonetheless welcome. The document, prepared by a committee under Dr Georges Carrier of Harvard University, was a key reference in the paper that remains the most coherent account of what the nuclear winter would be like, that of Turco *et al.* (*Science* 222, 1283-1292; 1983). Much of the delay has come about because of the diligence of referees of earlier drafts.

The nuclear winter is the phenomenon by which, in the aftermath of nuclear war, the upper atmosphere will be filled with particles of smoke from fires burning on the surface of the Earth. After a few days, the smoke will be distributed uniformly. If there is enough of it, the Sun will be obscured, the lower atmosphere cooled and a temperature inversion formed that may be stable for weeks or even months. Given persistence, and enough obscuration, surface temperatures would be so low for so long that most living things would die.

During the past year, the concept of nuclear winter has been widely canvassed. Apart from successors to the paper by Turco *et al.*, there have been public meetings, popular articles (of which the first seems to have been that by Carl Sagan of Cornell University in the supermarket magazine *Parade* for November 1983), and several speeches by politicians. Under the auspices of the International Council of Scientific Unions, the Scientific Committee on Problems of the Environment is well launched on a formal study of the problem that is due to be published next year.

It is in no sense surprising that the issue has been controversial. Most issues concerning nuclear war have this effect. Earlier reports on the consequences of nuclear war by the National Academy of Sciences, most recently in 1978, were deficient in their treatment of the consequences of atmospheric soot, a circumstance first pointed out by P.J. Crutzen and J.W. Birks (*Ambio* 11, 114-125; 1983). I have run into trouble with Turco *et al.* for suggesting, on grounds disputed, that their original article had been "hyped", as publishers say (see *Nature* 307, 107; 308, 11 & 311, 307; 1984), but I had not known then that the launching meeting had been supported by a grant of more than \$50,000 from the Boston-based Kendall Foundation to a public relations firm. Edward Teller made a similar case more

convincingly (see *Nature* 310, 621; 1984), if at greater length.

The National Academy's new report is a major contribution to this evolving discussion. (A full report of it will appear in *Nature* next week.) Inevitably, its effect is to accept that nuclear winter could be a consequence of a major nuclear exchange but to emphasize more clearly than has been customary the uncertainties in detailed calculations of what a nuclear winter would be like or how long it would last. Nobody will be surprised. In any attempt to predict the behaviour of a system as complicated as the atmosphere, it is natural that the starkness of first approximations should be relieved when refinements are introduced.

Those who think otherwise could do worse than glance at almost any issue of the purple version of the *Journal of Geophysical Research*. The October issue, for example, contains an account of one of the most ambitious attempts to calculate the climatic consequences of increased carbon dioxide in the atmosphere (Washington, W.M. and Meehl, G.A. *J. geophys. Res.* 89, 9475-9503; 1979). The authors have used the versatile community climate model they have helped to develop at the National Atmospheric Research Center (NCAR).

The calculation is both an illustration of how competent the climate modellers have become and of how far they have to go. The authors have set out to calculate the properties of two atmospheres, one with the present concentration of carbon dioxide, one with twice as much. The surface of the Earth is given a "realistic geography", which means that the continents are accurately placed where they should be, so that the variation of albedo over the surface of the Earth is not very different from reality. But the oceans are represented only by a slab of water 50 metres thick, which allows for seasonal heat exchange but not for global heat transport, a simplification which is sensible enough when the objective, as here, is simply to calculate the equilibrium condition of two atmospheres containing different amounts of carbon dioxide.

This version of the model is persuasive. It reproduces well enough the seasonal variation of the atmosphere as it is. Stratospheric temperatures come out well, for example, as do the general patterns of surface temperature and even wind velocity. Precipitation is less well calcu-

lated, sea-ice cover is over-estimated (perhaps because of the neglect of global oceanic transport), as are summer continental temperatures (because of the modellers' endless problem of allowing accurately for clouds).

The prediction of what the climate would be like if the carbon dioxide were twice as abundant as at present is in the circumstances all the more telling. Briefly, the greatest differences of temperature are at the poles, but also at mid-latitudes, while precipitation is everywhere increased. Inevitably, to the modellers, calculation has been seriously constrained by the speed with which even NCAR's computing resources can handle climatic fluctuations from one season to another. And this, in a sense, is merely an equilibrium calculation. Rapidly changing systems should be inherently more difficult to calculate accurately, although it may then be possible to omit some slowly-changing components of the climatic model.

Schneider *et al.* have shown (*Nature* 308, 21; 1984) that a different version of the community climate model can indeed be used to simulate nuclear winter; they decided at the outset to take the properties of the atmospheric soot layer as given. But that, of course, is where much of the argument about nuclear winter centres. Will the layer of soot be uniform and, if not, will it be stable? How long, in any case, will it last? To ask these questions is not to suggest that the nuclear winter is a kind of hoax but rather that the nagging difficulties in the original account persist — the National Academy's report clarifies but does not answer them.

For what it is worth, the same conclusion seems to apply even to the more fully studied problem of carbon dioxide. The same issue of the same journal gives an account of accurate measurements carried out in the past decade at three Canadian stations of the atmospheric concentration of carbon dioxide. The detail is absorbing, but C.S. Wong *et al.* (*J. geophys. Res.* 89, 9527; 1984) also confirm what others have recently suggested, that the rate of increase of carbon dioxide (at 1.4 parts per million per year) is between a third and a half of the rate measured in earlier decades. The reasons for this change are entirely unknown, but presumably involve interactions with either the sea or the biosphere. Washington and Meehl's work is no doubt still valid, but it will be longer before it comes into its own.

John Maddox

Magnetosphere

First success for a space mission and a comet for Christmas

from D.J. Southwood and D.A. Bryant

THE first active phase of the Active Magnetospheric Particle Tracer Explorers (AMPTE) space mission is successfully accomplished. On 11 September 1984 and again on 20 September, lithium ions were released in the solar wind immediately upstream from the terrestrial magnetosphere, some 110,000 km from the Earth's surface. At a meeting of the AMPTE science team on 25–27 October at the Applied Physics Laboratory in Laurel, Maryland, the data from the releases were assimilated and cross comparisons were made between the three spacecraft involved in the mission.

AMPTE is a joint project of the United States, West Germany and Britain. Each national team has provided a spacecraft. The US Charge Composition Explorer (CCE) carries a comprehensive set of instrumentation to detect the mass and charge composition of the terrestrial ionized environment, the magnetosphere. Its task is to monitor whether test ions released by the German Ion Release Module (IRM) can penetrate to its orbit. The IRM is also fully instrumented to make plasma and field measurements. The third spacecraft is the British subsatellite, UKS, added to the programme at the behest of the German team, who recognized that the actual pick-up of the release ions by the surrounding plasma posed a significant scientific problem in its own right. The UKS orbits close to the IRM so that both spacecraft are able to monitor the local effects of the ion releases.

The first result of the release experiment in the solar wind is a null one; the team are convinced that no lithium ions made their way from the release site to the CCE, deep in the magnetosphere. However, no disappointment was evident in the team, in part because of their exhilaration at the unique measurements made by IRM and UKS as the ions were created. In any case, the null result provides an important constraint on our understanding of solar ion access. Furthermore, the quality of the data from the passive measurements made routinely in the mission is so good that inevitably major gains will be made in our knowledge of the Earth's ionized environment and in its interaction with the solar wind.

The lithium releases produced artificial cometary effects. Comparison of IRM and UKS data show that the clouds initially formed a cavity in the solar wind from which the magnetic field was excluded. The scale of the cavity must have been well constrained because in both cases it contained IRM, whereas UKS saw no cavity in one case, and in the other only partial exclusion

of the field. The solar wind was slowed and diverted by the obstacle; in the process, the solar wind magnetic field was amplified. Magnetic forces led to the cloud being blown away from the vicinity of the spacecraft. Electron heating accompanied the field compression and the ion detectors recorded the signatures of classic cycloidal motion of the lithium ions as the solar wind picked them up and accelerated them. Further detailed plasma diagnostics were provided by plasma wave instruments. Beam instabilities, attributable to the interaction of lithium ions and solar wind protons, were detected but, during the passage over the spacecraft of the most intense electrical current sheets, no 'anomalous' (wave-induced) electrical resistivity seems to have been present.

Between the active ion-releasing phases of the mission, the three spacecraft make passive measurements of the Earth's ionized outer atmosphere. The measurements made during the magnetic storm of 4 September provide a high point of the

mission so far; CCE monitored the build up and decay of a hot plasma 'ring current' about the Earth, and IRM and UKS detected extreme solar wind, bow shock and magnetopause conditions.

The next ion release from IRM is due to take place on 25 December at 12.18 UT. The date was chosen for scientific reasons; the ideal configuration of the spacecraft and the ambient flow, combined with the requirements imposed by the position of viewing sites, constrained the team to two dates, of which Christmas Day is preferred to 27 December. The release will be of barium, which ionizes much faster than lithium, and will be made on the dawn flank of the magnetosphere, just outside the magnetopause. A longer-lasting and more substantial cavity is expected. The prime purpose is to create an artificial 'comet' which will be visible from North America and the eastern Pacific. The comet may be visible to ground observers (and other wise men) on the dawn side of earth for about twenty minutes.

Further releases of barium and lithium are planned for late March 1985. The ions will be released into the tail of the magnetosphere and CCE will be tracing the access of the ions to regions closer to Earth. □

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Gene regulation

Repression of activators

from Anna Velich and Edward Ziff

SINCE their first discovery in 1981, gene enhancers have increasingly been recognized as potent activators of transcription of a wide range of viral and cellular genes¹. A challenging report by Borrelli, Hen and Chambon on page 608 of this issue presents evidence of a mechanism of negative regulation of a viral enhancer². The finding is even more provocative because the proteins that Borrelli *et al.* show to repress enhancer activity are best known for their ability to activate genes. They are the E1a proteins of adenovirus-2, which are known to stimulate the transcription of both adenoviral and cellular genes in cells infected by the virus.

Enhancers are short segments of DNA that indirectly stimulate the transcription of nearby genes, perhaps by providing access for the transcribing enzyme, RNA polymerase II, to the genes. By contrast, the E1a proteins are direct activators of gene transcription^{3,4} by mechanisms that are unknown.

Chambon and his colleagues have studied the effect of E1a proteins on transcription of a plasmid containing a rabbit β -globin gene linked to the enhancer of polyoma virus. They show that when HeLa cells are transfected with this plasmid, the

polyoma enhancer stimulates transcription of the β -globin gene, but transcription is repressed if the cells are co-transfected with a plasmid that expresses E1a proteins. Unlike activation, the repression is independently effected by either of the E1a proteins. In competition experiments, additional enhancer DNA relieves repression by E1a, presumably by directly and competitively binding repressor. The E1a gene's own enhancer is active in this competition assay, suggesting that it may itself be repressed by E1a proteins and so be autoregulated. Chambon *et al.* show that the enhancer of simian virus 40 too is a target of repression, a result that is also indicated by data from our laboratory and which suggests that the repression of enhancers by E1a could be quite general.

Because, as the data show, activation and repression can be exerted under the same conditions, the E1a gene may command a complex control over transcription. With increasing production of E1a protein, a gene stimulated by it would be expressed and a gene dependent upon enhancers would be repressed. As the quantity of E1a protein falls, the activity of the E1a-dependent gene would dwindle, and the enhancer would regain its activity. The

activities of the two genes would be inversely related, with E1a providing a sort of 'flip-flop' control.

The focus on E1a proteins is of particular relevance because they are known to exert a profound effect on cellular metabolism, activating chromosomal genes for both heat shock protein⁵ and β -tubulin proteins⁶. E1a proteins have also been shown to activate certain other cellular genes in recombinant plasmids^{7,8}. In addition, as first reported by Houweling *et al.*⁹, if primary rodent cells are transfected with cloned E1a genes alone, they become immortalized. The mechanism of immortalization is unknown but may be the result of stimulation by E1a proteins of the same cellular proliferative functions that are stimulated by the proteins for the benefit of the adenovirus in infected cells. Perhaps the E1a proteins are mimicking the action of an unidentified endogenous stimulator of cell growth that is under the strict control of extracellular growth regulators.

Since the report of amino acid sequence homology between the E1a and myc oncogene proteins¹⁰ and the finding that either the E1a gene or the myc gene will complement a *ras* oncogene in transforming primary cells^{11,12}, any newly discovered property of myc or E1a is promptly sought in the other. Current evidence correlates myc gene expression with cellular proliferation, a feature perhaps akin to E1a's immortalization function. Myc production is transiently induced when growth factors stimulate resting fibroblasts or T cells to enter the G₁ growth state¹³⁻¹⁵. Regulation of the quantity of myc in the cell may also be important to differentiation. Many cell lineages maintain the ability to proliferate until they acquire markers specific for terminal differentiation, whereupon they withdraw from the cell cycle. During *in vitro* differentiation of the HL60 promyelocytic cell line, the expression of myc declines as the capacity for unlimited cell division is lost and differentiation markers are induced (ref. 16 and refs. therein).

Is there evidence for normal cellular proteins with the properties attributed here to E1a? Once again, a virus gives some useful information. The polyoma virus enhancer, shown by Borrelli *et al.* to be blocked by E1a, is inactive in undifferentiated embryonal carcinoma (EC) cells, but becomes active upon their differentiation (ref. 17 and refs. therein). Perhaps a protein in the undifferentiated cells represses the polyoma enhancer, as does E1a in HeLa cells, and perhaps this protein declines in quantity upon cell differentiation. Further evidence comes from the observation that class I major histocompatibility antigens are absent from undifferentiated EC cells but appear upon differentiation (ref. 18 and refs. therein). Provocatively, the E1a gene of the highly oncogenic strain of adenovirus, adenovirus-12, but not that of adenovirus-2, represses these antigens in transformed rodent cells¹⁹ and Borrelli *et al.* suggest that

their expression relies on an enhancer, which could be the target for adenovirus-12 E1a. Finally, EC cells do seem to have an E1a-like protein, which is indeed lost upon differentiation²⁰.

It is tempting to speculate that cellular proteins possessing activator and repressor functions analogous to those of E1a play a role in gene regulation during the growth and differentiation of cells. By inducing a high level of the proteins, a cell might turn on genes required for cell-cycling and repress other genes that are incompatible with proliferation but required in the resting state or after terminal differentiation. Could myc or a related protein provide such regulation? Experiments assessing any role for myc in gene transcription are scant, but there is a hint that, like E1a, myc can activate heat-shock genes²¹. And, as expected for an E1a-like protein, myc does decrease during EC cell differentiation¹⁴. Little is known about enhancer control, but the existence of tissue-specific enhancers^{22,23} makes it highly probable that there is regulation during differentiation. E1a's intriguing properties are directing us to fundamental features of the control of genes and, with time, all will be revealed. □

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Low-temperature physics

Do cosmic rays account for superfluid ³He transition?

from P. V. E. McClintock

IN THEORY, the transition between the two principal superfluid forms of liquid ³He ought not to be able to occur. So why is it routinely observed to take place? A possible answer to this intriguing question has recently been put forward by A. J. Leggett. Writing in *Physical Review Letters* **53**, 1096; 1984, he suggests that the transition, although prohibited from occurring spontaneously, readily takes place because of the occasional high energy cosmic ray that passes through any experimental chamber on the Earth's surface.

When helium gas is sufficiently cooled under atmospheric pressure, it eventually — albeit somewhat reluctantly on account of its very weak interatomic forces — condenses to form a colourless liquid. The liquid is of exceptional importance and scientific interest, enjoying the unique distinction of remaining a liquid down to the lowest attainable temperatures. There are two distinct forms of liquid helium, corresponding to the two stable isotopes, ³He and ⁴He. Liquid ⁴He undergoes a superfluid transition at about 2 K, entering a state in which a component of the liquid loses all its viscosity. It thus becomes able to flow quite effortlessly through holes or pores of vanishingly small dimensions; correspondingly, a small object travelling through the superfluid encounters absolutely no resistance to its forward motion. It

requires temperatures about 1,000 times colder for the same sort of behaviour, but in much more complicated form, to be observed in liquid ³He.

When liquid ³He is cooled under a pressure of more than about 20 atmospheres in a weak magnetic field, there is a very rapid onset of superfluidity at about 2 mK. The transition is second order, with no associated latent heat, and it always occurs at the same characteristic temperature for any given pressure. The superfluid phase that results is known as the A-phase. If the liquid is cooled further, it subsequently undergoes a first order transition, to the so-called B-phase superfluid. This transition is analogous to the more familiar phase changes of boiling or freezing, and occurs at a temperature that is not accurately reproducible. To put it another way, if the liquid is being cooled at a steady rate, the time taken for transition from A to B to occur is different on each occasion even if each experiment is carried out with the same cell and, so far as can be determined, in an identical fashion.

There is worse to come. In any pure system close to a phase transition, one can imagine small regions of opposite phase continually forming and disappearing again as the result of thermal fluctuations. Whether any one such region subsequently grows or shrinks will depend both on its

size and on the various bulk and surface energies of the two phases. Thus, $^3\text{He-A}$ could be cooled to a temperature where it would 'like' to become $^3\text{He-B}$, because the energy of the latter phase is lower, but cannot until a spontaneous thermal fluctuation produces a 'large enough' region of $^3\text{He-B}$. Large enough, in this context, means that the increase in the surface energy between the two phases, resulting from a small increase in the radius of the bubble of B-phase, is more than balanced by the corresponding decrease in energy due to the enclosed liquid being in the lower energy state: if this criterion is met, then the bubble of B-phase will obviously tend to expand until it has taken over the whole of the sample, whereupon the transition from A to B will be seen to have occurred.

It is possible to calculate the critical radius for the bubble of $^3\text{He-B}$ and hence, by the application of statistical mechanics, to estimate the average length of time before the transition occurs, given any particular degree of supercooling. Even on the most optimistic assumptions about the numerical values of the various parameters, the calculated result is a period of time vastly in excess of the age of the Universe. Hence, the transition should never be observed. And yet, even with a moderate degree of supercooling, the observed transition time is of the order of minutes.

Leggett's solution to this apparent impasse is based upon a consideration of the events that should follow the transit of a cosmic ray muon of about 2 GeV through the supercooled liquid, as can be expected every few minutes. The immediate consequences are reasonably predictable on the basis of detailed studies of high energy particles in helium bubble chambers: a number of relatively low energy electrons would be produced, each of which would give up much of its kinetic energy through the production of heat, initially creating a region of a few hundred Å in radius at a few tenths of a K, while the bulk of the liquid remained at its ambient temperature of 1–2 mK. What would happen next is a matter of some conjecture. Leggett's view is that an expanding shell of heated liquid would be created, propagating out from each original event, with the enclosed liquid cooling towards its ambient temperature. He points out that this 'baked Alaska' distribution would provide ideal conditions for the formation of B-phase ^3He as the heated liquid cools through the superfluid transition, and that the cooling would be too rapid for the expansion and domination of any small bubbles of A-phase ^3He that might be nucleated at the same time. From the point of view of the experimenter, unaware of the passage of the initiating cosmic ray, the transition from A to B will seem to have taken place spontaneously, a few minutes after the regime of a supercooled A-phase has been entered.

Now that Leggett's suggestion has been

aired, it should not be too difficult to set up suitable particle detectors and a coincidence counting system to test whether the transition coincides with the passage of a cosmic ray through the liquid. If the hypothesis is correct, it will open the way to study the metastable A-phase right down to near-zero temperatures in weak magnetic fields — a matter of considerable experimental and theoretical interest. All

that should be necessary is to shield the sample from cosmic rays, perhaps by conducting the experiments at the bottom of a deep mine. The first experimental test of Leggett's hypothesis will be awaited with very considerable interest. □

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Oncogenic intelligence

Cell immortalization and transformation by the *p53* gene

from D.P. Lane

A DIRECT role for the so-called *p53* gene of cells in the process of oncogenesis is suggested by three papers in this issue of Nature. Parada *et al.*¹ and Eliyahu *et al.*² both show that the protein encoded by the *p53* gene can complement activated *ras* genes in the transformation of primary rodent cells. The third report, from Jenkins *et al.*³ establishes that the *p53* gene can also immortalize such cells.

The *p53* protein was first detected in the form of its tight non-covalent complex with the large T protein of simian virus 40 (SV40)⁴. Subsequently it was found also to complex to the E1b 58K protein of adenovirus⁵. Though not closely related in structure, both these proteins are involved in the oncogenic action of their viruses, implying that their shared ability to bind the *p53* protein is important for their action in cellular transformation. The *p53* protein in primary cells and established non-transformed cells has a very short half-life and is present in minute concentrations, with each cell containing only a few hundred molecules. When bound to either viral protein, the half-life of *p53* is greatly extended and the protein accumulates, reaching concentrations of the order of 10,000 molecules per cell. Many transformed cell lines and primary tumour isolates also contain elevated levels of the protein^{6,7}. Microinjection of antibodies to *p53* into the cell nucleus of normal quiescent cells prevents their stimulation by serum, implying an important natural role for the protein⁸.

The new discoveries are provocative because they suggest that the alterations in *p53* levels and stability in many mouse and human tumours may be directly involved in their altered growth. Parada *et al.* introduced a cloned *p53* gene (linked to the murine leukaemia virus LTR) into primary rat embryo fibroblasts (REF) and Rat-1 cells. While the *p53* gene alone failed to transform either cell type, when it was introduced together with an activated *ras* gene, foci were produced in the REF cultures. Since the *ras* gene alone was also unable to induce foci, the *p53* gene seems to provide a complementation function in this

assay in much the same way as the cellular *myc* gene, adenovirus E1a gene and the large T gene of polyoma virus^{9,10}. Parada *et al.* further establish that cells transfected with both *p53* and *myc* genes give rise to tumours in nude mice. In both the focus-forming assay and the tumorigenicity test, the *p53* gene appears to be less efficient than the *myc* gene.

Eliyahu *et al.* obtain broadly similar results with either REF cells or Chinese hamster embryo fibroblasts. Again, *p53*, like *myc*, complements an activated *ras* gene in focus-forming assays. Interestingly, this group had some difficulty in establishing cell lines from the foci resulting from introduction of *p53* and *ras* genes together, and obtained evidence to suggest that overproduction of the *p53* protein can be very toxic to the cells.

Jenkins *et al.* go on to prove that *p53* can immortalize cells, thus increasing the strength of the similarity between *p53*, *myc* and E1a. The Wistar adult xiphisternum chondrocyte cells they used have a doubling-time of greater than 60 hours and undergo 30 doublings in culture before senescence and cessation of growth. But after transfection with a plasmid that contained the *p53* gene, early passage cultures of the cells had an extended life (200 doublings so far), a shorter doubling-time in serum, and the ability to be transformed efficiently into tumorigenic cells by an activated *ras* gene.

Since *p53* regulation is altered in so many human tumours, an obvious first priority, now is to look for alterations in the *p53* gene in human neoplasia. But deeper issues can also be addressed. Does SV40 large T have the unusual ability to transform primary cells both readily and directly because it first immortalizes by stabilizing *p53* and then induces morphological transformation by some other *ras*-like activity? If so, why are E1a proteins, rather than the *p53*-stabilizing E1b 58K protein, the immortalizing proteins of adenovirus? Moreover, why do certain small amino-terminal fragments of SV40 large T that are unable to complex *p53*, nevertheless have the ability to immortalize cells¹¹?

It is time to start looking more deeply for common attributes of all the immortalizing genes. Do others, apart from large T and *p53*, interact with each other and proceed through a common pathway? That some sophistication in our outlook is needed is highlighted by the recent characterization of an Abelson virus-transformed cell line, L12, that does not express *p53* because its gene has been inactivated by insertion of a retrovirus-like sequence into its first intron¹². L12 cells grow continuously in culture but, unlike other Abelson virus-transformed cells, will not give rise to lethal tumours in syngenic animals. Instead the tumours regress spontaneously despite reaching considerable size. When a functional *p53* gene is reintroduced into L12 cells, those cells which then express the gene are able to give rise to progressive and lethal tumours. So, in this system, the *p53* gene product seems to act as a tumour progression factor, whose effect is only really detectable in an *in vivo* assay. This should delight rather than mystify, because the

existence of a battery of oncogenes of subtly different effects, gives us a better chance to simplify and categorize the apparent plethora of human neoplastic diseases. □

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Immunology

Uses of chimaeric antibodies

from Alan Munro

ANTIBODIES were discovered through their ability to neutralize bacterial toxins, and for almost one hundred years antitoxin sera from experimental animals have been used to save many lives. For a brief period, it seemed that serum therapy would solve numerous medical problems. But it was soon found that repeated injections of a supposedly innocuous 'foreign' antibody can lead to harmful hypersensitivity reactions. With the availability of monoclonal antibodies, many new applications of serum therapy (for example, see last week's *Nature*; Cobbold *et al.* **312**, 548) would become well worth exploring if only hypersensitivity reactions could be avoided.

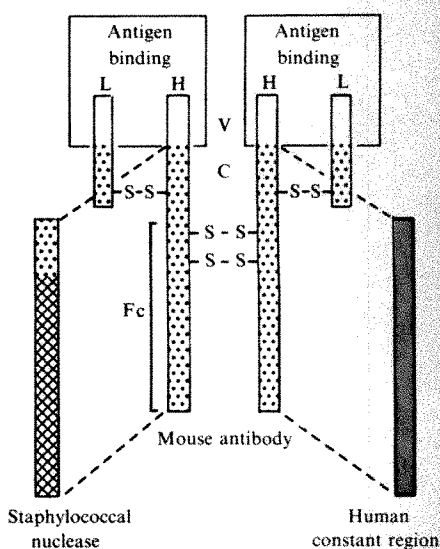
There are three ways to approach this problem. The first is to use monoclonal antibodies from the standard source — hybridomas of rat or mouse cells — but to induce specific unresponsiveness to rodent immunoglobulin before or during therapy. The second approach is to use human monoclonal antibodies, produced either from human hybridomas or from Epstein-Barr virus-immortalized human B lymphocyte cell lines; despite considerable efforts, the success of this approach has been limited and there is no generalized way of obtaining human monoclonal antibodies of the right specificity. Finally, by using the techniques of genetic engineering, it may be possible to obtain antibodies in which the antigen-binding site is defined by sequences from a rodent monoclonal antibody of the right specificity whereas the rest of the molecule is as 'human' in structure as possible.

Papers by G.L. Boulianne, N. Hozumi and M.J. Shulman on page 643 of this issue of *Nature* and by S.L. Morrison *et al.* in *Proc. natn. Acad. Sci. U.S.A.* **81**, 6851; 1984 are the first to describe functional chimaeric antibody molecules containing light chains and heavy chains, in which the whole of the variable regions of the chains are 'mouse' and the constant regions are 'human'. Possibly the human immune system will not recognize such chimaeric molecules as foreign, but there are good reasons to think that it will. In many cases it is possible to mount an immune response against antibody molecules themselves, even when the only antigenic determinants lie in the variable regions (take, for example, the heavy-chain variable region allotypes in rabbits). At least it may be easier to induce unresponsiveness to rodent variable regions when they are part of chimaeric antibodies. In that respect, it will be interesting to compare the rates of clearance of chimaeric molecules with those of the normal antibodies molecules of the host. Even if it proves possible to construct chimaeric antibodies with rodent hypervariable regions embedded in a human variable region framework, it is likely that they will be recognized by the human immune system — but the hope is that such chimaeras will be sufficiently 'self-like' to become integrated into the network of interacting idiotypes and anti-idiotypes without sensitizing the host and so avoiding subsequent hypersensitivity reactions.

The construction of chimaeric antibodies has been made possible through the

development, by a number of independent groups, of vector systems that allow the expression of antibody light-chain or heavy-chain genes in cells that have been transfected with them. Many laboratories have since initiated experiments in which cells are transfected with modified antibody genes. Earlier this year J. Sharon *et al.* (*Nature* **309**, 364; 1984) reported the expression of a novel fragment of antibody consisting of a normal light chain ($V_L C_\lambda$) associated with a modified antibody chain made up of a heavy-chain variable region linked to a light-chain constant region ($V_H C_\lambda$). This fragment binds antigen and is secreted from the transfected cells (showing that the Fc portion of antibody is not necessary for secretion).

The report by M.S. Neuberger, G.T. Williams and R.O. Fox on page 604 of this issue not only confirms that result but goes much further. It shows that the Fc portion of the antibody heavy chain can be replaced by a completely unrelated protein — the enzyme staphylococcal nuclease for example — to produce a bifunctional



Schematic diagram of a mouse antibody showing two identical light chains (L) and two identical heavy chains (H). Each chain consists of variable (V) and constant (C) domains. The antibody class is determined by the heavy-chain constant region which imparts distinctive features on the tail (Fc) region. Neuberger *et al.* have substituted staphylococcal nuclease for the Fc region of both heavy chains of immunoglobulin M; Boulianne *et al.* have replaced the C domain of each heavy chain of the immunoglobulin M monomer with a human C domain.

chimaeric molecule — in this case, exhibiting both specific antigen-binding and nuclease activity. There are many potential applications of this technology in the purification of cloned gene products, in enzyme-linked immunoassays and in the targeting of chemotherapeutic drugs on to tumour cells. □

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Cosmology

Strings and the origins of galaxies

from Neil Turok and David N. Schramm

THE idea that superheavy 'strings', formed at a phase transition in the very early Universe¹⁻⁴, provide an explanation of the origin of galaxies, has been the subject of much discussion (in part in these columns: see *Nature* 310, 365; 1984). We should like to take the discussion further as it is now becoming apparent that strings can make galaxies in ways quite different from other methods and thus may help solve or remove the dark-matter problems.

Strings are predicted to be formed at symmetry-breaking phase transitions in the very early Universe by those grand unified theories (GUTs) in which the vacuum has the appropriate topology^{5,6}. They have well-known analogues in condensed matter physics: for example, the flux lines formed in superconductors. Much as line defects in a crystal, they would form a network across the whole space. The relevant GUTs predict that strings would be formed at a temperature of about 10^{15} GeV at a cosmological time of about 10^{-35} seconds and with a mass per unit length of about 10^{20} kg⁻¹.

In some theories, strings can be formed after a period of inflation. They would be stretched by the subsequent expansion of the universe, and waves on a given scale would begin to oscillate as that scale entered the particle horizon. Whenever the string crossed itself, an exchange of partners could occur and produce closed oscillating loops of string with long lifetimes⁷. The gravitational field of these loops would cause matter to accrete around them⁸. Thus these strings could be the primordial density fluctuations needed in the early universe to explain the eventual formation of galaxies⁹. One point we wish to emphasize and show to be of interest is that, in such models, small loops are cut off from lengths of string which in turn are cut off as larger loops, and so on — in other words, the model creates real spatial correlation between density fluctuations of all sizes.

Until the advent of GUTs and inflation, primordial fluctuations were inserted into cosmology in a completely *ad hoc* way. With GUTs, a fluctuation spectrum with equal power on all scales forms naturally. This is thought to be an excellent way to produce the distribution of sizes in the Universe¹⁰. However, in the normal generation and application of such a spectrum, it is assumed that there is no spatial correlation between the large-scale and small-scale clumps. They each have a random probability (random phase) of occurring anywhere. On the other hand, strings still produce the same spectrum of sizes but also enable a correlation of large scales and small scales.

Galaxy formation with various forms

of dark matter has become an extremely active area of study¹¹. From big bang nucleosynthesis¹² it is known that baryons account for less than 15 per cent of the critical cosmological density. Observations of the dynamics of galaxies suggest that the matter clustered with galaxies is less than 40 per cent of critical density and the lower end of the observed range is consistent with the baryon limits. Thus the dynamical dark matter could be baryons; and some of it must be^{12,13}. There are, however, two arguments which drive us to look for other candidates. The first is inflation (see Guth in ref. 11) and the second is the limit on the anisotropy of the 3K background (see Usson and Wilkinson in ref. 11). Inflation is the only known way of explaining several of the otherwise extraordinary initial conditions of the Universe. But, barring fine tuning, inflation requires a critical density for the Universe; thus at least 85 per cent of the Universe cannot be baryons, and more than 60 per cent of the matter of the Universe did not cluster with galaxies. From the current limits on the anisotropy, Vittorio and Silk (in ref. 11) and Bond and Efstathiou (also in ref. 11) argue that the density of matter in the Universe must be greater than the baryonic upper limit.

To make things even more difficult, it has been noted that the spatial correlation function of rich clusters of galaxies has revealed strong clustering on very large scales (up to 150 Mpc)¹⁴. This correlation function is about 18 times stronger than the spatial correlation function for galaxies⁹, while varying as the same power (-1.8) of the spatial separation, r . (The correlation function is the excess probability over random that an object will be found within radius r of another.) It has also been found that the largest scales in the Universe seem to look filamentary, with large voids and large clumps (see Davis in ref. 11).

Different proposals have been put forth to try to solve each problem, but no model solves them all as long as it is assumed that the primordial fluctuation spectrum has random phases. For example, a model based on low mass neutrinos produces both critical density and large-scale structure (filaments, voids and cluster-correlation function) but does not account for the early formation of galaxies¹⁵. Models invoking heavy- or slow-moving particles (GeV mass photinos, gravitinos, axions or planetary-mass black holes) fit the small-scale structure, galaxy-correlation function, formation times and so forth¹⁶, as well as building hierarchically to yield clusters, but do not allow critical density to be reached. Even hybrid models, with both low-mass and high-mass 'inos', run into problems because the low-mass particles smear out

the small-scale structure. Some of us have even resorted to invoking heavy particles that decay to light ones, but this requires a lot of 'fine tuning'; others (see Bardeen in ref. 11) have suggested solutions where most clumps of matter do not shine as galaxies, thus making galaxies poor tracers of matter and negating the relevance of observational astronomy.

A more natural solution may be the non-random phases of string models. Independently, Peebles¹⁷ and we have noted that non-random phases may help. Such a model yields large-scale filaments and voids as the superheavy strings attract galaxies and clusters, and gives strong cluster-cluster correlations. Recent work¹⁸ has also shown that a model based on clustering of galaxies about filaments fits the higher (3- and 4-point) correlation-functions for galaxies as well as the hierarchical clustering paradigm. Such a model also enables density growth in some areas without producing a large universal background anisotropy and so could enable baryons to be the dark matter on galaxy and cluster scales, with non-baryonic stuff being a critical density background.

The degree of random to non-random phases in such models depends on the density of strings in space. In the limit of space being completely filled with strings, the string-picture also gives random phases. Even if string densities are large enough to randomize phases, their mere existence would still alter galaxy-information calculations, because the strings, rather than the matter, would carry the fluctuations, so standard arguments would not apply. For example, even if the fluctuations are carried by random-phased strings, neutrino streaming would no longer damp out small scales and so the low-mass neutrino model would no longer have problems with galaxy-formation times. To get the correlation function for galaxies to be less than that for clusters means that the fluctuations cannot be totally correlated, so the string density must be moderate. Numerical simulations of the motion of strings¹⁹ and of their spectrum at formation²⁰ suggest that the number of isolated loops falling inside the horizon should be only a few percent of the

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number either formed in association with, or subsequently produced by, the self-intersection of lengths of strings; perhaps ≥ 5 per cent of galaxies should be non-isolated. (Note that even an isolated string will behave differently from a random phase initial perturbation and it will be interesting to see how N-body simulations of galaxy formation are affected.)

Recently, there has been discussion of ways to observe cosmic strings using gravitational lensing effects or to establish the part strings may play in active galactic nuclei^{4,21,22}. Out of this may emerge direct

methods for resolving whether or not strings are present in today's Universe. Although much remains to be done to see if strings are relevant to galaxy formation, they provide an interesting supplement to exotic particles and provide cosmologists with a mechanism for producing non-random phases which totally alter traditional procedures of calculation. □

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Palaeontology

Consensus on archosaurs

from Michael J. Benton

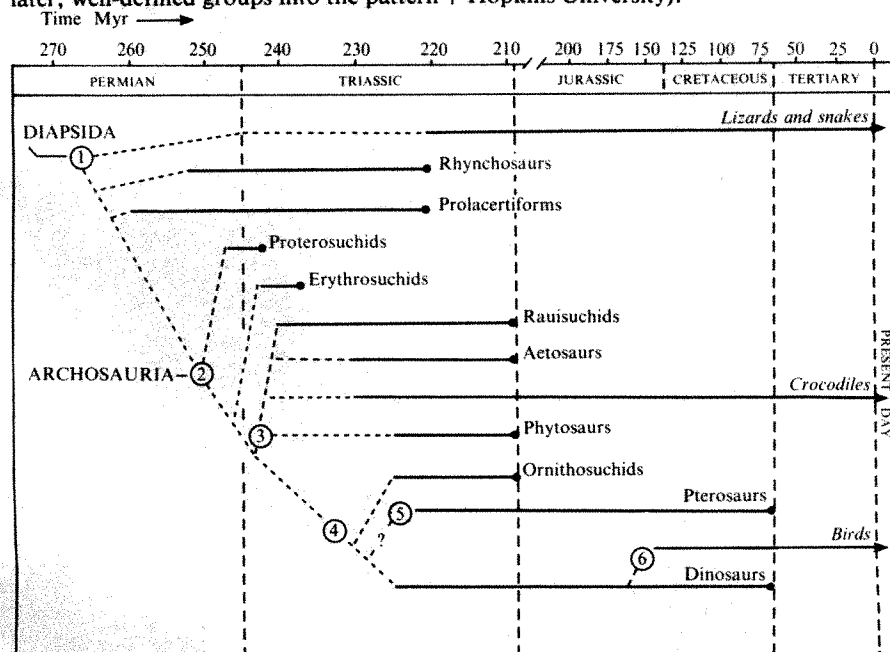
OBSCURITY shrouds the origins of the archosaurs, those diverse, scaled and feathered forms that include pterosaurs and dinosaurs, as well as the living crocodiles and the birds. Commonly, each line is traced back into a rag-bag group of early archosaurs of the Triassic period (208–245 Myr) called the thecodontians. The archosaurs are thought to have arisen just before the Triassic, but the origin and nearest relatives of the group have been obscure. Clarity and a radical new consensus on some of the issues were the outcome of the 3rd symposium on Mesozoic Terrestrial Ecosystems, held at Tübingen, FRG from 6–10 September, 1984.

The reason for the remarkable level of agreement in a hitherto obscured area of evolutionary biology was that each contributor applied the techniques of cladistic analysis to the relationships of the basal thecodontians, and only then slotted the later, well-defined groups into the pattern

obtained. Previous analyses had relied upon the assessment of general resemblances and the time sequence of the fossils in an attempt to pinpoint ancestors. The cladistic technique assumes that it is very unlikely (but not impossible) that we shall ever find an ancestor, and it concentrates on identifying nearest relatives — sister-groups — by an analysis of shared derived characters.

The radical new conclusions that were reached at the Tübingen meeting are best explained by reference to the numbered sequence on the summary tree below.

(1) The archosaurs form part of a larger group, the Diapsida, which also includes the living lizards and snakes^{1,2} (for a dissenting view, see ref. 3). Within the diapsids, the nearest sister-groups to the archosaurs are the rhynchosaurs and the prolacertiforms^{1,2} (J. Gauthier, University of California, Berkeley and G. Paul, Johns Hopkins University).



A tree (cladogram) representing the evolution of the archosaurs and based on the consensus of opinion at the Tübingen meeting. The tree is set against a timescale based on the known fossil record. Note the change in time scale at 200 Myr, which gives greatest emphasis to events in the Triassic.

(2) The early Triassic archosaurs — the Proterosuchidae and the Erythrosuchidae — are much more primitive than all later archosaurs, and are placed as basal sister-groups within the Archosauria; the later forms split into two major lines, one leading to the crocodiles, the other to the dinosaurs (M. Parrish, University of Chicago; Gauthier).

(3) The 'crocodile line' includes several specialized thecodontian groups of the middle and late Triassic: the superficially crocodile-like fish-eating phytosaurs, the snub-nosed herbivorous aetosaurs, and the carnivorous rauisuchians. The first crocodiles are known from the late Triassic. They were long-limbed lightly-built terrestrial forms, that evolved aquatic specializations after the extinction of the phytosaurs.

(4) The dinosaurs, as we currently understand that term, form a monophyletic group — that is, they all evolved from a single common ancestor, which was a late Triassic dinosaur. This important conclusion was agreed by Gauthier, Paul⁵ and Parish, as well as by D. Norman (University of Oxford), P. Sereno (American Museum of Natural History), and myself. Already in the 1970s, several palaeontologists had suggested that the dinosaurs were a monophyletic group^{4,5}, but this view had not been widely accepted. The new consensus is that the dinosaurs were a single group of advanced archosaurs sharing a number of derived characters of the limbs that were related to the animals' particular kind of upright stance (see *News & Views*, *Nature* 310, 101; 1984).

(5) More controversially, Gauthier and K. Padian (University of California, Berkeley) argued that the pterosaurs — the extinct flying reptiles — were the closest sister-group of the Dinosauria. Pterosaurs had forearms, bodies and heads specialized for flight but their hindlimbs are apparently very dinosaur-like.

(6) Finally, there was much more agreement than seemed possible a year or so ago (see *News & Views*, *Nature* 305, 99; 1983) on the relationships of the birds. This was touched on briefly at the Tübingen meeting, but was discussed in greater depth at a special meeting devoted to the 'early bird' *Archaeopteryx* held in Eichstätt, FRG. Nearly everyone now accepts that the birds arose from the dinosaurs, and from the bipedal carnivorous dinosaurs (the theropods) in particular. There was little support for the alternative view that the birds sprang directly from the Triassic thecodontians, or from a crocodile-like ancestor, and the discussion was more about which theropod family was most closely related to the birds. □

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Geology

So you thought mountain ranges were complicated

from Myrl F. Beck

REMEMBER the time when it was thought that the major features of the Earth's crust were riveted in place as securely as armour plates on a battleship? It took courage to be a tectonic theorist, but life may have been easier for the quadrangle-mapper. In those days, crustal blocks mostly moved up and down, or perhaps a few tens of kilometres laterally at most, so one naturally looked for solutions to local geographical problems in one's own backyard. If granitic debris suddenly appeared in a sedimentary section, it meant uplift of the granitic batholith immediately across the valley. There was no need to complicate life by looking any further.

Even with the advent of early plate-tectonic theory the quadrangle-mapper's task was still much the same; unless his map area spanned a major suture, the several parts of his study area could still be assumed always to have been close together, barring a few highly unusual, and geologically easily recognizable, circumstances. But time has complicated this simple picture. In the past decade, a combination of geology and paleomagnetism has shown that at least one orogenic belt (the North American Cordillera) is a moraine of crustal fragments, many with oceanic affinities, transported intact from points of origin hundreds or thousands of kilometres away. Geological studies showed that many adjacent Cordilleran crustal blocks are too unlike one another in stratigraphy and structural history to have evolved in juxtaposition, and that some of these crustal blocks, or terranes, are wholly exotic to North America. To settle this issue, paleomagnetism has provided dramatic evidence of ultra-long-distance transport of crustal blocks.

This microplate model is familiar to many geologists. Orogenesis and the growth of the continent are held to have been more the result of collision and off-scraping of these prefabricated crustal elements than of subduction of the oceanic plates upon which they rode. The model also holds that, once attached to North America, many of these terranes were disrupted by strike-slip faulting; the resulting fragments are now distributed along the continent's edge, placed there by a process that might be termed tectonic longshore drift. Large rotations about vertical axes also may occur during this stage. Because subduction along the western edge of North America has been north-oblique since at least the late Mesozoic, transport and rotation in the Cordillera seem to have been mostly northward and clockwise, respectively. The scale of the process is

hotly debated; some pieces of the Cordilleran mosaic are as small as a house, but others clearly are at least as large as, say, Vancouver Island.

Nevertheless, it still seems to be true that mountain belts are a product of plate interaction, and that they are built at plate margins, usually at the edge of a continent. It should follow that major events in the tectonic history of mountain belts reflect major changes in the behaviour of plates. It should, therefore, be possible to investigate the nature of orogenic cause-and-effect by observing correlations between important tectonic transitions in a mountain belt (as from overthrust to extensional faulting) and events along the plate margin (as, for instance, a change from rapid to slow convergence).

But a mountain belt is an exceedingly complex recorder. Tectonic events overwrite earlier deformations on the same piece of crust without completely erasing the earlier record — thereby making both records hard to interpret. Also, continental crust in an orogenic belt varies enormously in thickness, age, and physical properties from place to place; it seems hopelessly optimistic to assume that the same orogenic response will always follow a particular plate-tectonic event. The timing of tectonic events is a further problem. Such 'events' last several millions or tens of millions of years, and most are complex and progressive — that is, they involve several geological processes acting simultaneously or in sequence. It is hard to know when an 'event' actually starts or stops. Thus, correlations with plate-tectonic phenomena are bound to be difficult to make, and often will not be particularly convincing.

Still more trouble arises because of the nature of the record of relative plate motions. In the Cordilleran example, three oceanic plates (Farallon, Kula, Pacific) and one continental plate (North America) are involved. According to most plate models, direct Pacific–North America interaction has been a factor in Cordilleran tectonics only for the last 30 Myr or so. The chief culprits have been the Farallon and Kula plates, of which, respectively, very little and nothing remain. We deduce the relative motion histories of these two plates from what we hope are mirror-image anomaly patterns recorded on the Pacific plate — thereby putting ourselves explicitly at the mercy of the symmetrical spreading hypothesis of plate tectonics. Likewise, many models make use of the trends of the Hawaiian Islands and the Emperor Seamount chain to deduce absolute motion of the Pacific plate. Any inaccuracy in the

notion that hotspots are fixed relative to each other thus transfers directly to our plate reconstructions. Finally, it has recently been shown that inaccuracies in specifying stage poles for finite rotations increase with the age of the pole, and may become quite large. This means that some of our Mesozoic reconstructions may be seriously in error.

But where was the Kula–Farallon–North America triple junction during the late Mesozoic and early Tertiary? Since the relative motions of Kula–North America and Farallon–North America were quite different at times, the triple junction ought often to have separated regions of distinctly different tectonic style. It may have moved up and down the coastline rather erratically, however. Perhaps the geology will help us locate the triple junction and thereby improve the plate models. The tectonic consequences of transferring large exotic terranes from the oceanic to the continental crust are poorly known; in particular the effect on plate motions is uncertain.

Whether or not the Cordilleran microplate model can be applied to other mountain belts remains to be seen. For the present, pity the poor quadrangle-mapper. No longer can he assume that the batholith across the valley was there when his sedimentary section began recording its influx of granitic debris; without definite paleomagnetic or geological evidence to the contrary, it might instead have been part of, say, Sumatra. □

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100 years ago

The Edible Mollusca of Great Britain and Ireland. With Recipes for Cooking Them. By M. S. Lovell.

THE primary object of the author is to call attention to the qualities and merits of many kinds of shell-fish which are as nutritious as others which are generally known, but which are rarely met with in our markets, or are only used locally for food, while the proper modes of cooking them are scarcely known. Accordingly all the known species of edible shell-fish on our coasts are here described in succession. This alone would make the volume of great use at a time when we are going to the uttermost ends of the earth for the sources of our food-supply. But when we add that the writer has collected from the most varying sources a mass of curious lore about shell-fish, their uses, and the mode of catching them in various parts of the globe, their medicinal properties, the popular superstitions about them, &c., it will be perceived that this is much more than a work on natural history *plus* a cookery-book. Of all the many uses of snails, the strangest perhaps is that discovered by the London adulterator. They are much employed, the author assures us, in the manufacture of cream, being bruised in milk and boiled, and a retired milkman pronounced it the most successful imitation known!

From *Nature* 31, 124, 11 December 1884.

Direct observations of the dopant environment in fluorites using EXAFS

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Calcium fluoride (CaF₂), like the important UO₂ phase, is a defect system having the remarkable property that large concentrations of trivalent rare-earth dopants can be accommodated within its structure with no loss of structural integrity. Using extended X-ray absorption fine structure (EXAFS), we have observed the way in which the local structural environment of the impurity alters with the size of the rare-earth ion and can distinguish, by computer lattice simulation studies, between dimer and hexamer configurations for light and heavy dopants, respectively.

THE structural properties of anion-excess CaF₂ have been intensively investigated during the last 20 years. Many of the most important problems in the study of non-stoichiometry are raised by these systems which have simple structures and for which well characterized samples are available. Diffraction has been used previously to study the more heavily defective alkaline earth fluorides but is inherently limited when applied to disordered solids as it provides only average structural information. In contrast, extended X-ray absorption fine structure (EXAFS), which yields information on the local structural environment of individual atom types^{1,2}, is uniquely able to distinguish between the complex defect cluster structures proposed for the phase.

The rare-earth trifluorides are highly soluble in the alkaline-earth fluorides. The fluorite structure, typified by CaF₂, can readily accommodate F interstitials which compensate for the charge due to substitutional trivalent dopants. The structure can be easily visualized as a simple cubic array of anions with half the cube centres occupied by cations; the remaining vacant cubes provide the interstitial sites, as illustrated in Fig. 1a.

For low dopant concentrations (≤ 0.1 mol %), it appears that dopants are isolated and that their charge-compensating anions occupy the adjacent cube-centre sites forming nearest-neighbour pairs³. As the dopant concentration increases, evidence from laser spectroscopy⁴ and neutron diffraction^{5,6} suggests that dopant aggregation occurs, forming clusters with two or more substitutional rare-earth ions and their accompanying interstitial F ions. Various cluster structures have been envisaged containing 2–6 dopant ions, and their relative stabilities calculated by lattice simulation^{7,8}. One cluster involving a pair of dopants is shown in Fig. 1b. Stability is achieved by relaxations of the dopant and interstitial F ions in a $\langle 110 \rangle$ direction, towards the central cube edge, accompanied by a relaxation of the nearest pair of lattice anions outwards, in the $\langle 111 \rangle$ direction. The structure is catalogued as a $2[0]2[2]_1$ cluster. This designation uses a systematic notation for clusters of the type $i|v|p|q_r$, in which i denotes the number of substitutional dopant ions, v the number of vacancies, p the number of relaxed lattice ions and q the number of interstitials in nearest-neighbour sites (for which $r=1$)⁸. Recent calculations predict that this cluster will readily trap a free interstitial fluoride ion, to form the relatively more stable, negatively charged $2[0]2[3]_1$ cluster (Fig. 1c). The early neutron diffraction work of Cheetham *et al.*⁹ on Y-doped CaF₂ found evidence for the low-symmetry interstitial configuration predicted by the $2[0]2[2]_1$ model. The more recent diffraction work of Catlow *et al.*⁵ supports the formation of the $2[0]2[3]_1$ in La-doped CaF₂. However, several workers have suggested the formation of larger cubo-octahedral clusters^{10,11}: Figure 1d shows the hexameric $6[0]8[6]_1 \langle 111 \rangle$ cluster, which the calculations show to

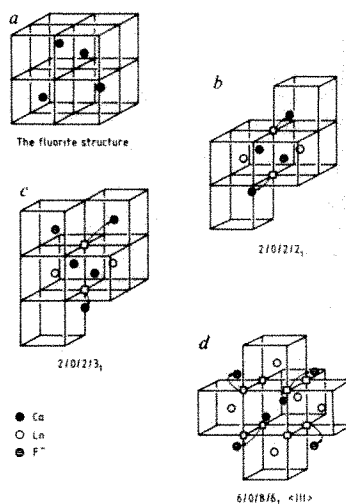


Fig. 1 a, The fluorite structure. b, The $2[0]2[2]_1$ dopant dimer cluster. c, The $2[0]2[3]_1$ dopant dimer cluster. The arrows show the postulated relaxation pathways of the lattice F ions. d, The $6[0]8[6]_1 \langle 111 \rangle$ dopant hexamer. (Five of the six lanthanide dopants, and four of the F interstitials, sited opposite the edges of the central cube, are drawn in d. The arrows show how a 45° rotation of the central cube faces, followed by an outward relaxation, will generate 8 of the 14 F interstitials.

be the most stable of the clusters containing six dopant ions.

Detailed calculations of the relative energies of the different clusters discussed above revealed that the stability of the hexameric cluster, compared with that of the smaller dopant dimers and tetramers, increased markedly as the dopant ion radius decreased. Thus, at the high Z end of the lanthanide series, simulations predicted that the larger hexameric clusters will predominate. On the other hand, at the low Z end of the series, calculations suggested that no one particular cluster—dimer or hexamer—is preferred; the larger hexameric clusters should become the preferred structure at Gd in the series where the radius has dropped to 1.05 Å (ref. 8).

Some support for a change in cluster structure with dopant radius has been found in the neutron diffraction studies^{6,7} which show significant differences between interstitial structures in La-doped CaF₂ and Er-doped CaF₂. However, as noted, diffraction gives only average structural information; EXAFS, on the other hand, provides information on the local environment of the dopants and should give direct evidence for such a change in cluster structure. Furthermore, detailed structural models

Table 1 Radial distances in Ln/CaF₂ structures

No. of atoms	Type	RD from EXAFS (Å)	Debye-Waller factor σ^2 (Å ²)	Simulation RD (Å)
a				
10	F	2.40	0.014	2.40
1	Nd	3.97	0.013	3.79
11	Ca	3.91	0.024	3.87
1	F	4.18	0.005	4.05
24	F	4.50	0.028	4.53
6	Ca	5.45	0.026	5.46
24	F	5.95	0.030	5.95
24	Ca	6.69	0.035	6.69
b				
9	F	2.35	0.011	2.35
1	F	3.32	0.007	3.45
8	Ca	3.93	0.016	3.83
2	F	4.12	0.007	4.05
4	Er	4.21	0.023	4.05
22	F	4.54	0.034	4.55

Comparison of radial distances (RD) obtained from fitting: *a*, the Nd/CaF₂ data to the 2[0]2[3] cluster configuration, and *b*, the Er/CaF₂ data to the 6[0]8[6] cluster configuration, with the corresponding distances predicted by computer simulations.

from computer simulations are especially useful in interpreting the EXAFS of these disordered crystalline solids.

EXAFS spectra

Figure 2 shows the EXAFS spectra for the Ca_{0.9}Ln_{0.1}F_{2.1} series; Ln is an element in the rare-earth series La to Yb. In traversing this series, the frequency of the EXAFS oscillations decreases and the shape of the fine structure is modified: in particular, the shoulder on the peak around 50 eV shifts to lower energies, a shoulder appears on the high-energy side of the peak around 100 eV and the feature at 125 eV disappears. Qualitatively, the local structural environments of Gd–Yb in CaF₂ are essentially the same but differ from those displayed by La–Nd. Thus, the EXAFS data support the predictions of the computer simulations of a change in preferred structure about midway across the lanthanide series.

By taking the Fourier transform of the experimental data, it is possible to obtain a distribution function, similar to a radial distribution function, for the atoms surrounding the rare-earth ion^{12,13}. The difference between the two types of local structure surrounding the rare-earth defects can be seen in Fig. 3, where the Fourier transforms of Nd/CaF₂ and Er/CaF₂ are compared with that of Ca in CaF₂. All three distributions display three separate peaks in the range 0–5 Å. Whereas the structure surrounding Ca persists out to 10 and 11 Å, that surrounding the rare-earth dopants is less extensive. Nd displays some features beyond 5 Å at similar distances to those in CaF₂ but the atomic environment of Er is less well ordered. The histograms in Fig. 3 represent the radial distributions of atoms surrounding dopants for the most stable clusters predicted by the lattice simulation studies^{7,8} compared with that surrounding Ca in CaF₂. In all three environments, there are predominantly three shells in the range 0–5 Å which can be used to identify the peaks in the experimental EXAFS atomic distributions: 8 or 9 fluorines at 2.3–2.4 Å, 11 or 12 cations (Ca/Ln) at ~4 Å and a further 20–24 fluorines at ~4.5 Å. It is immediately obvious from Fig. 3 that the peaks for the second and subsequent shells of neighbours in the EXAFS distributions are much weaker for Nd and Er than for Ca. For shells with similar atomic weighting, there can be little doubt that the local fluorite lattice is considerably distorted by the presence of rare-earth dopants.

Electron phase shifts

The central problem in calculating the EXAFS of a postulated spectrum is to obtain accurate estimates of the phase shifts of the photoelectrons. These are functions of the photoelectron

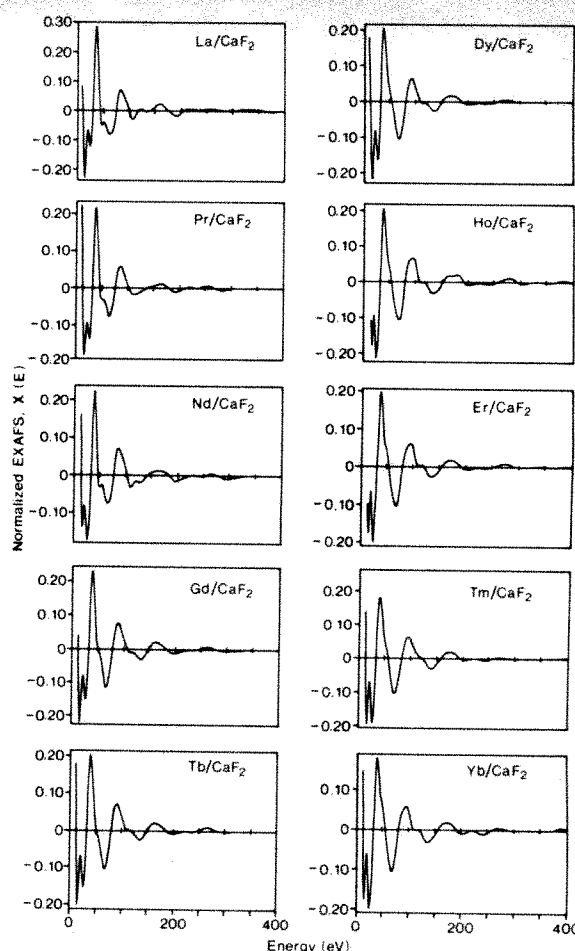


Fig. 2 The lanthanide, Ln(III), EXAFS for the series Ca_{0.9}Ln_{0.1}F_{2.1}. Single crystals of Ca_{0.9}Ln_{0.1}F_{2.1} were grown from 4N-purity powder LnF₃ (Rare Earth Products Ltd) and CaF₂ using a modified Stockbarger technique¹⁷. The rare earths used were Ln = La, Pr, Nd, Gd, Tb, Dy, Ho, Er, Tm and Yb. Portions of the single crystals were ground to a fine powder using a micronizing mill, then mixed with powdered polythene and pressed in a 13 mm die to form coherent pellets. The ratios of sample to polythene and pellet thicknesses were calculated so as to yield an absorption ratio, $\ln(I_0/I)$, of 1.20 at the energy of the rare-earth ion's Ln(III) absorption edge. The X-ray energies of the lanthanides used cover the range 5.49–8.95 keV. The EXAFS measurements were performed at room temperature in transmission mode, using the EXAFS station 7.1 on the SERC Synchrotron Radiation Source (SRS) at Daresbury. The SRS ran at 2 GeV with a typical beam current of 100 mA. An order-sorting two-crystal monochromator was used. Data analysis was carried out using the EXAFS program package on the Daresbury Laboratory NAS 7000 computer.

wave vector $\mathbf{k}$ (the momentum of the photoelectron ejected in the absorption process), and are related to the normalized EXAFS (χ) by the expression:

$$\chi(\mathbf{k}) = \sum_j A_j(\mathbf{k}) |f_j| \sin(2kR_j + \psi) \quad (1)$$

where each of the j shells of neighbours contributing to the EXAFS pattern has an amplitude term, A_j , depending predominantly on the number of atoms in that shell; R_j is the distance from the central atom to the atoms in the j th shell; and ψ is the total shift in phase imparted to the escaping photoelectron wave in passing through the atomic potentials of the central atom and the j th shell atoms. The amplitude function, $|f_j|$, describes the effectiveness of the atoms in the j th shell in backscattering photoelectrons of different momenta and can be calculated from the phase shifts^{14,15}. Thus, to determine the atomic positions accurately, it is necessary to obtain reliable phase-shift functions over the entire energy range of the EXAFS

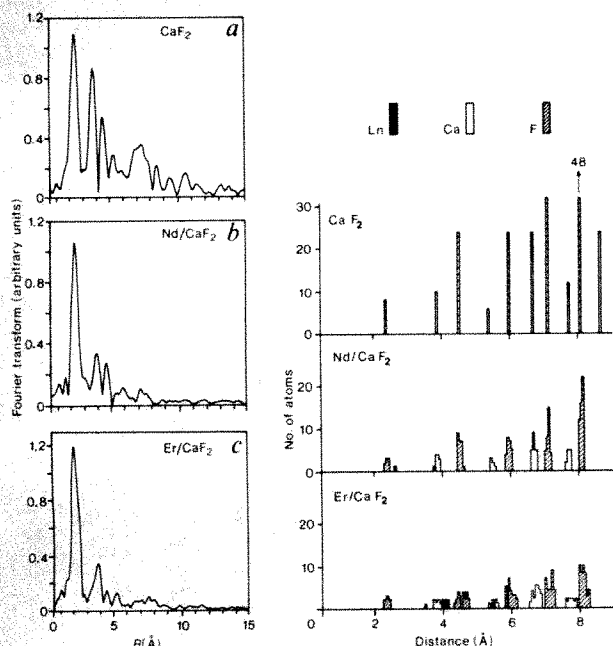


Fig. 3 Fourier transforms of the experimental data of: *a*, K edge of Ca in CaF₂; *b*, Ln(III) edge of Nd in Ca_{0.9}Nd_{0.1}F_{2.1}; *c*, Ln(III) edge of Er in Ca_{0.9}Er_{0.1}F_{2.1}. Also shown are histograms depicting the radial distribution function of the three cations for CaF₂, and the computer simulations of the 2[0]2[3]₁ cluster for Nd/CaF₂ and the 6[0]8[6]₁(111) cluster for Er/CaF₂.

spectrum. In the simplest case, the total phase shift for a particular atom pair (for example, Ca and F) can be obtained empirically using model compounds for which the local geometries are known¹⁶. Alternatively, multiple phase shifts can be calculated for individual atoms¹⁴. The main advantage of using calculated phase shifts is that the EXAFS of postulated structures can be calculated *ab initio* using the spherical-wave approximation, which is particularly important in analysing EXAFS spectra, such as for the fluorites measured here, where the energy range of discernible fine structure (0–400 eV) is limited. The principal disadvantage, however, is that the chemical components in the phase-shift functions are difficult to reproduce precisely. For example, phase shifts for Ca in a metallic environment will be slightly different from those in an ionic environment and the difference is difficult to calculate realistically from first principles.

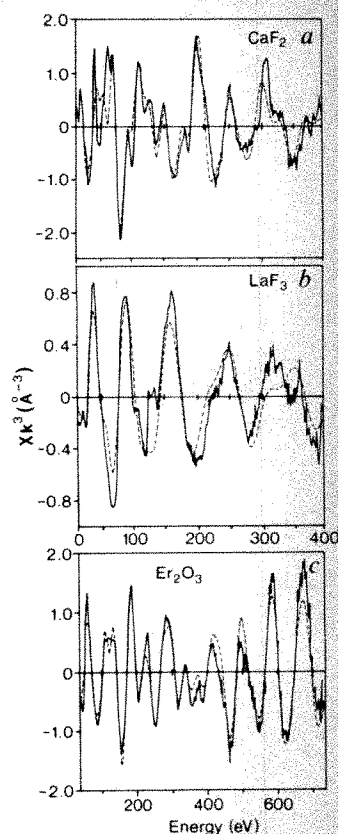
For these reasons, we have taken calculated phase shifts as starting parameters and have used least-squares methods to adjust their magnitude and *k*-dependence until the best fit to the experimental data for several model compounds is achieved (N. Binsted and L.M.M., in preparation). The structural standards chosen were CaF₂, Er₂O₃ and LaF₃ in which the cations are in similar electronic and chemical environments as the samples. Figure 4 shows the experimental data (weighted by *k*³ to enhance the oscillations at high energy) and the calculated EXAFS obtained using these phase shifts and the appropriate coordination numbers. The analysed shell radii for neighbouring atoms up to 5 Å from the central atom agree with crystallographic values to better than 0.03 Å. All three spectra display the characteristic complex beating indicative of the contribution to the EXAFS of many shells of neighbouring atoms, as can be seen in the Fourier transform of the CaF₂ data (Fig. 3a).

Lattice simulations

To test whether the observed transition in the lanthanide EXAFS series presented in Fig. 2 corresponds to a change from a dimer to a hexameric cluster, we have calculated the theoretical fine structure profile expected for the different structures.

Previous simulations^{6,7} predict that the distance from the rare-earth ion to its nearest fluoride neighbours is less in the

Fig. 4 Fits to structural standards for *a*, CaF₂; *b*, LaF₃; and *c*, Er₂O₃. —, Experimental data with *k*³ weighting; ---, theory. All fits give the correct radial distances to within 0.01 Å for neighbours out to 4.5 Å. Subsequent radial distances are within 0.15 Å of the correct distances. This discrepancy can be attributed to multiple scattering and core-hole relaxation effects.



hexameric clusters than in the smaller aggregates. This may be the driving force for the smaller lanthanides at the high atomic-weight end of the series to form these larger clusters. Another difference between the computed cluster structures lies in the second shell consisting of 12 Ca ions for pure CaF₂; for the dimeric clusters the second shell has 11 Ca ions and 1 rare-earth ion, whereas for the hexamers, 4 out of the 12 cations are dopant ions (Fig. 3).

Nd/CaF₂ and Er/CaF₂ exemplify the two types of EXAFS observed across the rare-earth series (Fig. 2). Figure 5 shows the *k*³-weighted spectra for each: note that the fine structure is less detailed than for CaF₂ (Fig. 4), which is symptomatic of the increase in disorder introduced into the lattice by the presence of the dopant rare-earth cations, as noted before when the Fourier transforms were compared.

In analysing the Nd/CaF₂ and Er/CaF₂ spectra, we observe a shortening of the first-shell radius. Figure 6 shows the inverse Fourier transform of the filtered first peak of each spectrum. The frequencies are clearly different. The Er/CaF₂ spectrum has the lower frequency and hence the shorter first-shell radius. The Ln–F distances determined using the corrected phase shifts are 2.40 Å and 2.35 Å for Nd/CaF₂ and Er/CaF₂, respectively. The first-shell radii predicted by lattice simulation calculations consist of 10 fluorines at 2.40 Å for the dimeric 2[0]2[3]₁ cluster and 9 fluorines at 2.35 Å for the 6[0]8[6]₁(111) cluster.

The second important difference between the dimeric and hexameric clusters is in the second shell where there are one Ln and 11 Ca for dimers but for hexamers there are 4 Ln and 8 Ca. The amplitude of the backscattering factor for Ca is quite different from that of Ln so different occupancies of the cation shell around 3.9 Å will result in different fine structure. We find that this is indeed the case and is responsible for most of the differences between the measured spectra. Further evidence in support of larger rare-earth ions preferring smaller clusters is the enhanced contribution to the EXAFS from the third-shell F ions in the case of the Nd/CaF₂ spectra. The peak at ~4.5 Å in the Fourier transform is larger for Nd/CaF₂ than for Er/CaF₂; this is expected as larger clusters cause longer-range disordering with a concomitant increase in the variance of the value of the radial distance. This is reflected in an observed increase, for

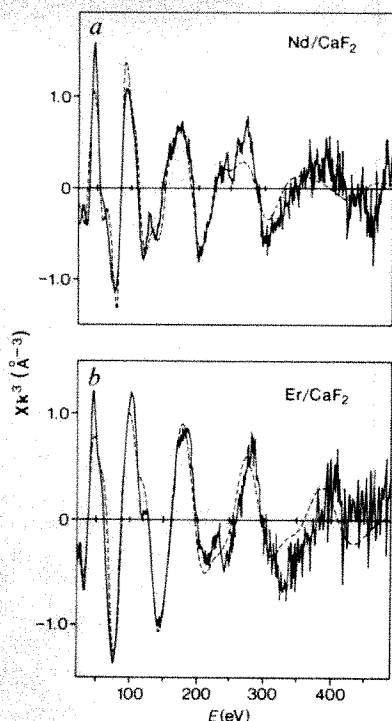


Fig. 5 The k^3 -weighted Ln(III) EXAFS of: *a*, $\text{Ca}_{0.9}\text{Nd}_{0.1}\text{F}_{2.1}$ and *b*, $\text{Ca}_{0.9}\text{Er}_{0.1}\text{F}_{2.1}$. The broken line shows the calculated EXAFS obtained using the parameters listed in Table 1.

Er/CaF_2 , of the Debye-Waller factor (see Table 1). This term contributes to A (equation 1) and accounts for loss of amplitude due to static as well as any thermally induced disorder.

The dashed curves in Fig. 5 demonstrate how the detailed differences between the dimer and hexamer cluster structures reproduce the observed differences in the EXAFS spectra of Nd and Er-doped CaF_2 . The theory reproduces the changes in structure of the peaks around 50 eV and 150 eV, including the

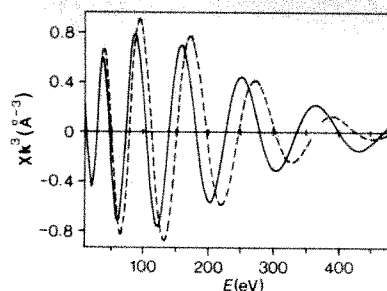


Fig. 6 A comparison of the Fourier-filtered first shell of F neighbours for Ln(III) EXAFS of $\text{Ca}_{0.9}\text{Nd}_{0.1}\text{F}_{2.1}$ (—) and $\text{Ca}_{0.9}\text{Er}_{0.1}\text{F}_{2.1}$ (---).

minimum around 140 eV. The fine structure between 200 and 300 eV is also accurately reproduced for the two structures. Details of the coordination number, shell radius and Debye-Waller factors are given in Table 1. Our results are in good agreement with the lattice simulation calculations. However, the accuracy in determining the radial distance to shells containing only one or two atoms is less than for shells having higher occupancies, owing to the smaller contribution of the former to the total spectrum. Lattice simulation calculations predict that the stabilization of the different clusters in the lighter lanthanides is very similar, leaving the possibility that a mixture of different clusters could be formed. Certainly, returning to Fig. 2, there are some small differences in the measured EXAFS amongst the La, Pr and Nd spectra, but the analysis of the Nd EXAFS spectrum (Fig. 5*a* and Table 1*a*) suggests that the presence of aggregations larger than the dimeric cluster is unlikely for the lighter lanthanides. Differences between the spectra for the heavier lanthanides are more subtle, in accordance with the greater stability of the hexameric cluster predicted by the calculations.

We thank the SERC for a grant (GRB/57484), the Director and Staff of Daresbury Laboratory for the provision of synchrotron radiation and associated facilities, and N. Binsted at Daresbury for expanding the EXAFS data analysis codes.

Received 10 July; accepted 24 October 1984.

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Recombinant antibodies possessing novel effector functions

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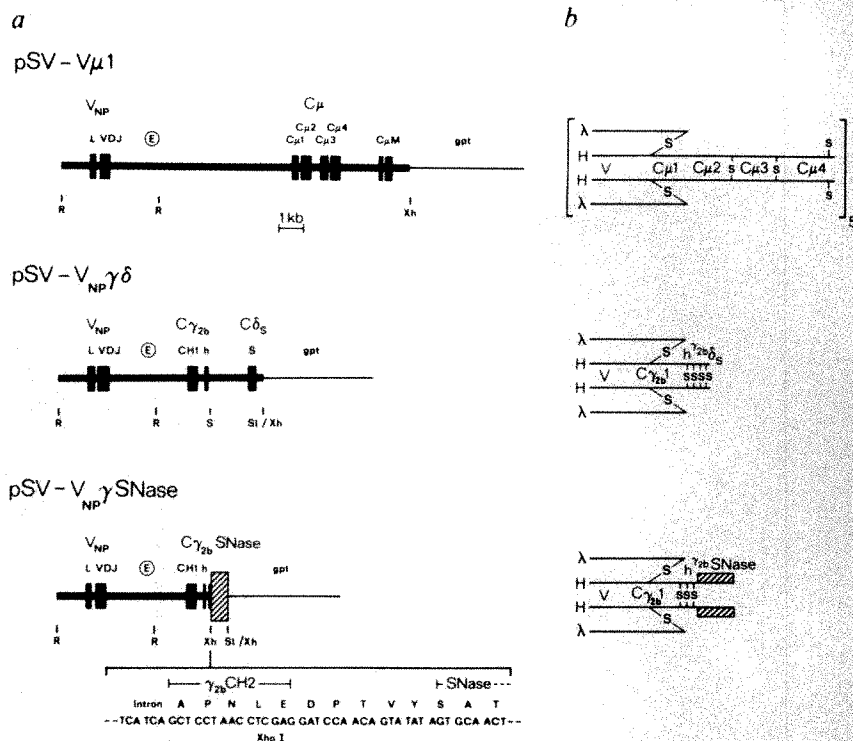
The introduction into lymphocytes of immunoglobulin-gene DNA that has been manipulated in vitro allows the production of novel antibodies. In this way, cell lines have been established that secrete hapten-specific antibodies in which the Fc portion has been replaced either with an active enzyme moiety or with polypeptide displaying c-myc antigenic determinants.

MONOCLONAL antibodies secreted by hybrid myeloma cells¹ are used extensively both as diagnostic reagents² and in therapy³. Recent techniques allowing the stable introduction of immunoglobulin-gene DNA into myeloma cells⁴⁻⁶ make it possible to use *in vitro* mutagenesis and DNA transfection to produce

recombinant antibodies possessing novel properties. In particular, chimaeric proteins in which the antigen-binding portion of the immunoglobulin is fused to an enzymatic moiety could be of use in immunoassays. Furthermore, the chemical coupling of toxins to cell-specific antibodies has been advocated as a

Fig. 1 **a**, Structure of plasmids. Thin horizontal lines, pSV2gpt vector; thick lines, immunoglobulin-gene DNA; boxes, exons; hatched box, *S. aureus* nuclease coding region. The locations of the heavy-chain locus transcription enhancer element (E) and the *gpt* gene are indicated. Restriction endonuclease cleavage sites: R, *EcoRI*; Xh, *XhoI*; S, *SacI*; and S1/Xh, a sequence formed by joining a *SacI* to an *XhoI* site. The sequence is presented around the *XhoI* site of plasmid pSV-V_{NP}γSNase, which forms the junction of the γ2b C_{H2} exon and the SNase gene. **b**, Predicted structures of the anti-NP antibodies whose heavy chains are encoded by the plasmids in **a**. Disulphide linkages (-S-) between heavy (H) and light (λ) chain are indicated. Only one bivalent subunit of the decavalent pSV-V_{NP}γδ-encoded IgM is illustrated.

Methods: In all three plasmids, the V_{NP} exons are contained on a common *EcoRI* fragment and the vector is the *BamHI*-*EcoRI* fragment of pSV2gpt (ref. 12) with an *XhoI* adapter in the *BamHI* site. pSV-V_{NP}γδ contains an *EcoRI*-*SacI* and pSV-V_{NP}γSNase an *EcoRI*-*XhoI* mouse Cγ2b fragment derived from phage λMYG9 (ref. 13). Thus pSV-V_{NP}γδ contains the γ2b C_{H1} and hinge exons whereas pSV-V_{NP}γSNase also contains the 5' end of the C_{H2} exon. The mouse C_{δs} exon of pSV-V_{NP}γδ is contained in a *BamHI* fragment of phage Ch257.3 (ref. 14) which was obtained as a *SacI*-*SacI* fragment after subcloning in M13mp11. The SNase coding region derives from an M13mp8 clone containing a *Sau3A* fragment of *S. aureus* DNA inserted in the *BamHI* site; this clone was isolated from an M13 library by antibody screening (R.O.F., unpublished data). The DNA encoding the five amino acids linking the γ2b C_{H2} domain and SNase derive from the SNase leader sequence. Removal of the SNase gene from the M13mp8 clone as a *BamHI*-*SacI* fragment and recloning in M13mp12W (ref. 15) allowed its isolation as a *XhoI*-*SacI* fragment for final assembly of pSV-V_{NP}γSNase.



technique in chemotherapy⁷; antibody fusions containing cytotoxic carboxy-terminal moieties could be exploited in this area.

Myeloma cells provide an attractive system for the production of chimaeric antibodies, as such cells are specialized in the synthesis and secretion of large amounts of immunoglobulin. However, at present, we do not know whether chimaeric antibodies can be secreted by a myeloma cell nor whether an antibody/enzyme fusion will retain both antigen-binding and enzymatic activity. We describe here experiments to test the feasibility of making recombinant antibodies in myeloma cells.

Reconstitution of hapten-specific antibody

We have described previously⁵ the construction of plasmid pSV-V_μ1 which includes a complete mouse immunoglobulin μ gene cloned in the expression vector pSV2gpt (Fig. 1). The immunoglobulin μ polypeptide encoded by this plasmid has a heavy-chain variable region, V_H, characteristic of λ1 light chain-bearing mouse antibodies that are specific for the hapten 4-hydroxy-3-nitrophenacetyl (NP); NP binding should therefore occur following association of the pSV-V_μ1-encoded heavy chain with mouse λ1 light chains. To confirm this, pSV-V_μ1 DNA was introduced by spheroplast fusion into J558L, a mouse plasmacytoma expressing λ1 but producing no heavy chain⁴. Stably transfected cells were selected as described in Fig. 2 legend and were cloned by limiting dilution. Twenty clones were analysed by radioimmunoassay and each secreted high levels of an NP-specific IgM antibody (data not shown). Homogeneous anti-NP IgM antibody can be purified from supernatants of pSV-V_μ1-transfected clones with a yield of about 3 mg l⁻¹ (Fig. 2).

Secretion of F(ab')₂-like antibody

To test whether deletion of the Fc portion of the heavy-chain gene results in impaired antibody synthesis or secretion, a derivative of pSV-V_μ1 was constructed in which the exons encoding

the μ-chain constant region (C_μ) were replaced by the C_{H1} and hinge exons of the mouse γ2b gene. To provide translation termination and polyadenylation sequences, an exon, C_{δs}, derived from the gene encoding secreted mouse δ chains, was placed at the 3' end of the gene (Fig. 1). The plasmid containing this truncated heavy-chain gene, pSV-V_{NP}γδ, would be expected to direct the synthesis of a F(ab')₂-like molecule consisting of two IgG2b Fab molecules disulphide-linked via the γ2b hinge and with a 21-amino acid tailpiece at the carboxy-terminus encoded by the C_{δs} exon.

Plasmid pSV-V_{NP}γδ was transfected into J558L cells and radioimmunoassay revealed that the stably-transfected cells secreted high levels of λ1-bearing anti-NP antibody. This NP-specific antibody was purified from culture supernatants of several transfected clones with a yield of 5–10 mg l⁻¹. Polyacrylamide gel electrophoresis of the purified material in non-reducing conditions (Fig. 2c) reveals a major protein species of relative molecular mass (*M_r*) ~110,000, which agrees well with the predicted *M_r* of a F(ab')₂-like molecule. Minor bands of *M_r* ~50,000 are also observed which may be from Fab' molecules. On reduction, a band co-migrating with λ light chain and several polypeptides of higher *M_r* are detected; the major band among these has an *M_r* of 31,000 and could constitute the heavy chain of the F(ab')₂-like antibody.

The minor antibody heavy-chain components do not reflect glycosylation heterogeneity, because the electrophoretic mobilities of the pSV-V_{NP}γδ-encoded heavy-chain bands are unaffected by inclusion of tunicamycin in the incubation medium during biosynthetic labelling experiments (Fig. 2b). As the protein samples analysed were purified on antigen columns, it is likely that the minor species differ from the F(ab')₂ antibody in the carboxy-terminal portion of the heavy chain, possibly as a result of alternative processing of pSV-V_{NP}γδ transcripts.

Nevertheless, it is clear that F(ab')₂-like anti-NP antibody can be synthesized and secreted in high yield by pSV-V_{NP}γδ-transfected J558L cells. This agrees with previous observations⁸ that

Fig. 2 SDS-polyacrylamide gel electrophoresis of anti-NP antibodies secreted by J558L transfectants. **a**, Purified antibody (30 μ g) boiled with 2-mercaptoethanol before electrophoresis and stained with Coomassie blue; **b**, biosynthetically labelled antibody analysed after reduction. Samples purified from cells labelled in the presence of tunicamycin are indicated (Tm); **c**, unreduced antibody (30 μ g) purified from culture supernatants.

Methods: J558L cells stably transfected with the pSV plasmids were obtained by spheroplast fusion and selected essentially as described previously⁵ except that hypoxanthine-aminopterin-thymidine was omitted from the selective medium and mycophenolic acid was used at 5 μ g ml⁻¹, as described by Oi *et al.*⁴. Transfection frequencies of 10⁻⁴ to 10⁻³ were obtained: in all cases, cells were cloned by limiting dilution. Antibody samples were purified from supernatants of J558L transfectants grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Supernatants (2 l) were passed over 2-ml columns of 4-hydroxy-5-iodo-3-nitrophenacetyl-aminocaproic Sepharose (NIPcap-Sepharose) and antibody was eluted from the washed sorbent with 1 mM NIPcapOH in phosphate-buffered saline. Biosynthetically-labelled antibody was purified on 40 μ l NIPcap-Sepharose columns from supernatants of cells incubated for 4 h at 37 °C in medium containing L-³⁵S-methionine. Tunicamycin (8 μ g ml⁻¹) was, if required, included during a 2-h preincubation as well as during the labelling itself; parallel incubations with an IgE-secreting cell line confirmed the efficacy of the drug. Reduced samples were analysed on 12% polyacrylamide gels and 7% gels were used for unreduced samples.

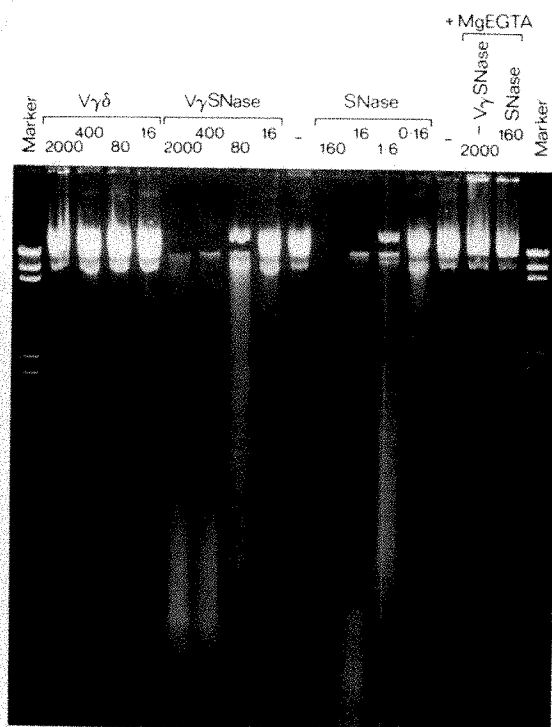


Fig. 3 Assay for nuclease activity. Single-stranded M13 DNA (2 μ g) was incubated at 37 °C for 30 min in 25 mM sodium borate, 250 mM NaCl, 10 mM CaCl₂, pH 8.5 (20 μ l) containing various amounts of V_{NP}γδ or V_{NP}γSNase antibody or of purified *S. aureus* nuclease. The quantities of antibody/enzyme used are in ng. DNA in the samples was then analysed by ethidium bromide fluorescence after electrophoresis through a 1.2% agarose gel. A HindIII digest of phage λ DNA provides size markers. Ca²⁺-dependency of the nuclease activity was confirmed by running incubations in the presence of 40 mM MgCl₂ and 25 mM EGTA.

monovalent recombinant Fab-like molecules can be secreted by transfected myeloma cells.

Secretion of Fab-nuclease

As a novel activity to fuse to the antibody heavy chain, we chose the nuclease from *Staphylococcus aureus*, selected because it is a secreted enzyme active as a monomer, contains no thiol

residues and can easily re-fold after denaturation⁹. A DNA restriction fragment containing the *S. aureus* nuclease (SNase) gene was inserted into the *Xho*I site located in the C_H2 exon of the mouse γ2b gene. Plasmid pSV-V_{NP}γSNase was assembled as described in Fig. 1 legend; the heavy-chain gene of pSV-V_{NP}γSNase is similar to that of pSV-V_{NP}γδ except that the C_δ exon has been removed and replaced by an exon containing the first five codons of the γ2b C_H2 exon fused in phase to the nuclease-coding region. Simian virus 40-derived sequences of the pSV2gpt-derived vector provide polyadenylation signals. J558L cells were transfected with pSV-V_{NP}γSNase and cells surviving in selective medium were cloned by limiting dilution.

Radioimmunoassay of supernatants of cloned transfectants revealed that about one-third were positive for the production of λ-bearing anti-NP antibody. Positive clones yielded 1–10 mg l⁻¹ of NP-binding antibody. Analysis of biosynthetically-labelled antibody by gel electrophoresis reveals a band comigrating with λ l light chain as well as two heavy-chain bands of *M*_r 45,000 and 46,000 (Fig. 2b). The difference between these two heavy chains has not been identified but their mobilities agree well with the predicted mobility of the V_{NP}γ-SNase heavy chain. Although the sequence Asn-Asn-Thr is present in SNase, the two V_{NP}γSNase heavy-chain bands are still present in samples purified from supernatants of cells that have been biosynthetically labelled in the presence of tunicamycin (Fig. 2). This demonstrates that the difference between them is not due to N-linked glycosylation. The V_{NP}γSNase antibody seems somewhat more heterogeneous on a non-reduced gel, giving bands with the expected mobilities of both the F(ab')₂-SNase and Fab-SNase. The presence of SNase on the heavy-chain carboxy-terminus might inhibit disulphide linking of the γ2b hinge regions.

Enzymatically active Fab-nuclease

To test for nuclease activity in the V_{NP}γSNase preparation, samples purified on hapten-Sepharose columns were incubated with single-stranded DNA substrate. Digestion of the DNA was monitored following agarose gel electrophoresis. V_{NP}γSNase but not V_{NP}γδ antibodies show nuclease activity and this activity, like that of authentic *S. aureus* nuclease, is dependent on Ca²⁺ but not Mg²⁺ ions (Fig. 3). As judged on a molar basis, the catalytic activity of the V_{NP}γSNase sample is ~10% that of authentic *S. aureus* nuclease.

The V_{NP}γSNase antibody can be used as a genetically conjugated enzyme-linked antibody in enzyme-linked immunosorbent

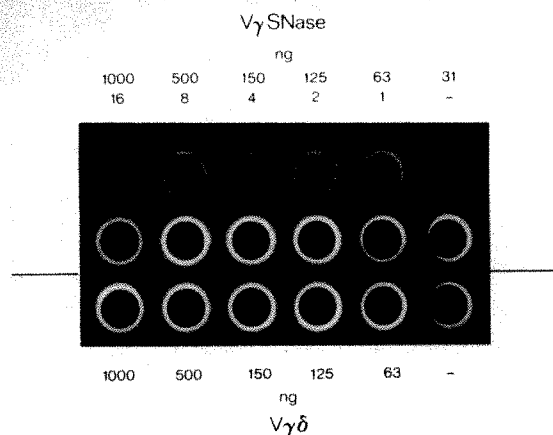


Fig. 4 Use of $V_{NP}\gamma$ SNase antibody in ELISA-type assay. Polyvinyl microtitre plates were coated with $40 \mu\text{g ml}^{-1}$ (NIP)₂₀-bovine serum albumin (BSA). After blocking unreacted sites with BSA, dilutions of $V_{NP}\gamma$ SNase or $V_{NP}\gamma\delta$ antibodies were incubated in the wells; ng of antibody incubated in each well are indicated. After washing off unbound material, a solution ($40 \mu\text{l}$) containing $1 \mu\text{g}$ M13 single-strand DNA and $1 \mu\text{g ml}^{-1}$ ethidium bromide in pH 8.5 buffer (see Fig. 3 legend) was added. The plate was photographed after 1 h incubation at 37°C .

assays (ELISAs). Antigen-coated plastic plates were incubated with various amounts of $V_{NP}\gamma$ SNase protein and bound antibody was then detected by its nuclease activity, by adding to the plate a solution containing DNA and ethidium bromide. Following digestion of the DNA substrate by the immobilized $V_{NP}\gamma$ SNase antibody, the fluorescence due to the DNA/ethidium bromide complex substantially decreased. Concentrations of $\sim 10 \text{ ng}$ $V_{NP}\gamma$ SNase antibody are detected easily; no decrease in fluorescence is obtained with the $V_{NP}\gamma\delta$ antibody control (Fig. 4). The assay may be made at least 10-fold more sensitive by increasing the incubation time with the DNA substrate.

Secretion of Fab-myc

As discussed above, the nuclease is an attractive moiety to fuse to an antibody because of its stability, ease of renaturation and

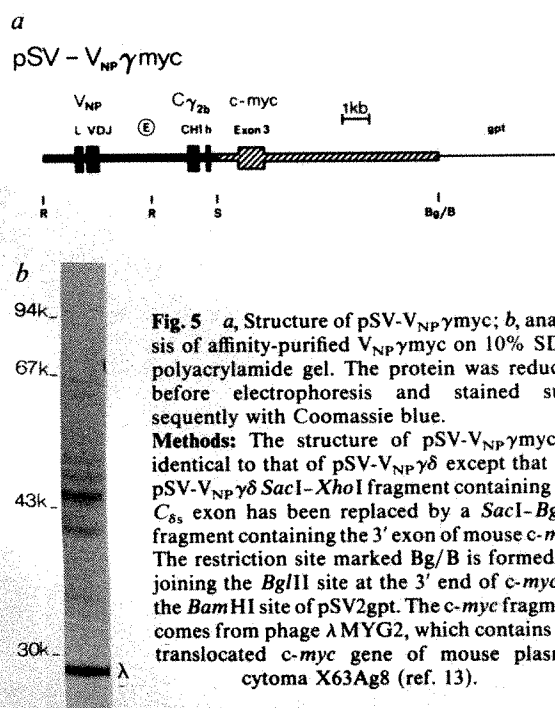


Fig. 5 a, Structure of pSV- $V_{NP}\gamma$ myc; b, analysis of affinity-purified $V_{NP}\gamma$ myc on 10% SDS-polyacrylamide gel. The protein was reduced before electrophoresis and stained subsequently with Coomassie blue. **Methods:** The structure of pSV- $V_{NP}\gamma$ myc is identical to that of pSV- $V_{NP}\gamma\delta$ except that the pSV- $V_{NP}\gamma\delta$ *SacI*-*XhoI* fragment containing the $C_{\delta s}$ exon has been replaced by a *SacI*-*BglII* fragment containing the 3' exon of mouse *c-myc*. The restriction site marked Bg/B is formed by joining the *BglII* site at the 3' end of *c-myc* to the *BamHI* site of pSV2gpt. The *c-myc* fragment comes from phage λ MYG2, which contains the translocated *c-myc* gene of mouse plasmacytoma X63Ag8 (ref. 13).

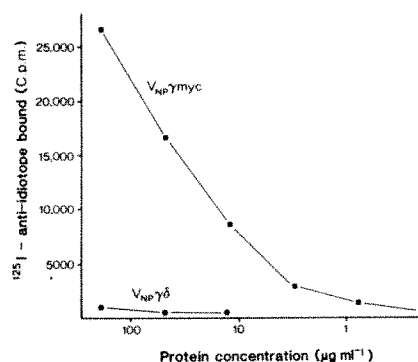


Fig. 6 Assay for *c-myc* antigenic determinant in Fab-*myc*. Monoclonal anti-*c-myc* antibody CT14-G4.3 (gift of G. Evan) was coated ($10 \mu\text{g ml}^{-1}$) onto polyvinyl microtitre plates and unoccupied sites were then blocked with BSA. Varying concentrations of either anti-NP Fab-*myc* (■) or $F(ab')_2$ (●) were incubated in the wells and, after washing, bound anti-NP antibody was detected by developing with radioiodinated monoclonal anti-idiotope antibody Ac38. The Ac38 antibody (gift of M. Reth and K. Rajewsky) recognizes the variable region of the anti-NP antibodies¹⁶. Controls performed with Ac38-reactive monoclonal IgM, IgD and IgG antibodies ($200 \mu\text{g ml}^{-1}$) gave less than 400 c.p.m.

lack of thiol groups and the fact that it is itself a secreted protein. We therefore decided to fuse the carboxy-terminal portion of the mouse oncogene *c-myc* to the antibody Fab to test the generality of the approach. The product of the *c-myc* gene is a protein which contains many thiol groups and normally exists in the cell. There is no reason to believe that the third exon of *c-myc* on its own will encode a functional protein domain. Thus, if the Fab-*myc* fusion protein were secreted by the cell, not only would it provide a source of protein for making anti-*myc* antisera, but also it would allow us to assess the general feasibility of secreting chimaeric antibodies.

Plasmid pSV- $V_{NP}\gamma$ myc was assembled as described in Fig. 5 legend; the plasmid is similar in structure to pSV- $V_{NP}\gamma\delta$ except that the $C_{\delta s}$ exon is replaced with the 3'-terminal exon of the mouse *c-myc* gene. This *c-myc* exon encodes 187 amino acids¹⁰ and should provide the transcription polyadenylation signal. The plasmid was transfected into J558L and cells from wells positive for production of anti-NP antibody were cloned by limiting dilution. Hapten-binding protein was purified from culture supernatants and analysed for the presence of *c-myc* antigenic determinants in an indirect radioimmunoassay. Samples of either the putative Fab-*myc* or the anti-NP $F(ab')_2$ (to act as control) were incubated in wells of a polyvinyl microtitre plate that had been coated with a monoclonal anti-*c-myc* antibody; bound anti-NP antibody was then detected using a radioiodinated monoclonal anti-idiotope antibody which recognizes the Fab portion of the anti-NP antibodies.

The Fab-*myc* clearly binds to the monoclonal anti-*c-myc* antibody, whereas the anti-NP $F(ab')_2$ and other controls do not (Fig. 6). The Fab-*myc* was also recognized by two other monoclonal antibodies specific for the carboxy-terminal end of *c-myc* (data not shown). SDS-polyacrylamide gel electrophoresis (Fig. 5) of the Fab-*myc* reveals that it is somewhat heterogeneous; a band co-migrating with λ 1 light chains and several bands with a higher M_r of 38,000–55,000 are observed, without a single dominant heavy-chain band being apparent. The expected size of the Fab-*myc* is M_r 50,000. We have observed that, after prolonged storage, precipitates appear in the Fab-*myc* sample and SDS-polyacrylamide gel electrophoresis reveals more extensive heterogeneity of the heavy-chain bands. We therefore believe that the heterogeneity of the Fab-*myc* protein indicated by the SDS-polyacrylamide gel analysis may be caused by proteolytic degradation.

Conclusion

Myeloma cells can synthesize and secrete active recombinant antibodies. The Fab-nuclease is secreted in good yield, retains enzymatic activity and is a stable protein. We have shown how such an antibody/enzyme fusion protein can be used in immunoassay and it is clear that analogous recombinant antibodies could find application in other fields. The results obtained with the Fab-*myc* construct suggest that although it may be possible to drive secretion of a large variety of different substitutions of the Fc portion, problems may be encountered with the stability of the resultant protein; in this case it is probable that the *c-myc*-encoded part of the antibody is unable to fold as a protein domain. Nevertheless, the fact that *c-myc* antigenic

determinants are clearly displayed by the Fab-*myc* protein suggests that this might be a valid approach to the preparation of antisera against the previously unidentified products of specific genes.

Finally, it is worth noting that these antibody/enzyme fusions may provide an attractive means for driving the synthesis and extracellular secretion of specific enzymes as fusion proteins which could then be purified easily on antigen columns. The enzyme and antibody moieties could, if required, be dissociated using specific proteases¹¹.

We thank S. Morrison for the J558L myeloma, Gerard Evan for the monoclonal anti-*c-myc* antibody and Graham Hatfull, Franco Calabi and Cesar Milstein for encouragement.

Received 10 September; accepted 30 October 1984.

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Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription

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The adenovirus-2 early region 1A (E1A) products repress activation of transcription induced by the simian virus 40, polyoma virus and adenovirus-2 E1A enhancers. The repression probably involves an interaction between the enhancer elements and a trans-acting factor(s), possibly the E1A products themselves.

• ENHANCERS are promoter elements, unique to eukaryotes, which potentiate transcription from other homologous or heterologous 'natural' or 'substitute' promoter elements (such as the TATA box), either in the presence or absence of upstream (distal) promoter elements (refs 1-5 and references therein). Viral and cellular enhancers exhibit species and/or cell specificity^{1,2,4,6-8}, suggesting that their activity may be positively regulated by factors acting *in trans*. Recent studies from our laboratory have shown that stimulation of transcription *in vitro* by the simian virus 40 (SV40) enhancer involves the formation of a stable complex between a *trans*-acting factor and the enhancer element⁹⁻¹¹. No example of the negative regulation of an enhancer element has yet been described. However, we found previously¹² that when HeLa cells were co-transfected with a recombinant containing the rabbit β -globin gene linked to the polyoma virus (Py) enhancer and a recombinant expressing the adenovirus-2 (Ad-2) early region 1A (E1A) gene products, there was a marked decrease in the amount of RNA initiated from the β -globin gene promoter. This suggested that the E1A products might block the activity of the Py enhancer. Such a possibility is intriguing, as it is known that the E1A products encoded in 13S and 12S messenger RNAs are involved in the activation of transcription from all other early viral transcription units¹³⁻¹⁵, as well as in cell immortalization and cell transformation¹⁶⁻¹⁹. Moreover, transcription from cellular promoters can be stimulated by the E1A products when recombinants containing these promoters are either co-transfected with the E1A transcription unit into HeLa cells or transfected into 293 cells that express

constitutively an integrated E1A gene (refs 20-22 and references therein).

We demonstrate here that the E1A products repress the activity of the SV40, Py and Ad-2 E1A enhancers, and that this inhibition probably involves an interaction between the enhancer elements and a *trans*-acting factor(s), possibly the E1A products themselves.

E1A products repress enhancer activity

We used the recombinant plasmid pE1ASV (Fig. 1; ref. 12), containing the entire E1A transcription unit including the enhancer sequences²³⁻²⁵, to study the effect of the E1A products on the transcription of two enhancer-containing recombinants. The first of these, p β (244+) β , contains the Py enhancer linked to two rabbit β -globin genes; the second, pSVBA34 (ref. 5), contains the SV40 enhancer (72 base pair (bp) repeat region) linked to a fragment of the Ad-2 major late promoter (Ad-2MLP) and the SV40 large-T antigen coding region (Fig. 1). When increasing amounts of pE1ASV were co-transfected into HeLa cells with a constant amount (10 μ g) of the p β (244+) β recombinant, a progressive decrease in RNA transcribed from the β -globin genes was observed (Fig. 2a, Glob +1; b, lanes 1-3). A sixfold decrease was obtained with 100 ng pE1ASV (Fig. 2a, lane 4), corresponding to a ratio of 1 molecule of pE1ASV plasmid to 30 molecules of the polyoma β -globin recombinant. In parallel experiments, the stimulation of transcription from the E2a transcription unit by the E1A products was assayed by co-transfecting pE1ASV and pE2G (a chimaeric recombinant containing

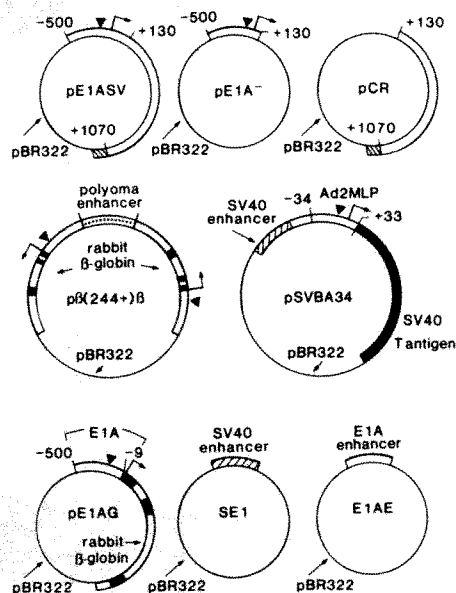


Fig. 1 Structure of the recombinants. pE1ASV (4.6 kilobases (kb), see ref. 12) contains the 1,570 left-most nucleotides of Ad-2. The E1A transcription unit from positions -500 to +1,070 with respect to the E1A cap site is linked to the 135-bp SV40 *Hpa*I (2,604)/*Bam*HI (2,469) fragment containing the SV40 polyadenylation signal (hatched box). pE1A⁻ (3.4 kb, see ref. 12) and pCR (4 kb) were derived from pE1ASV and contain respectively the E1A promoter region (positions -500 to +130) or the coding region (positions +130 to +1,070). pE1AG (5.2 kb, a gift of C. Goding and C. Kédinger) contains the E1A promoter from positions -500 to -9 linked by a *Hind*III linker to the rabbit β -globin gene (from positions -9 to +1,650 with respect to the β -globin cap site). p β (244+) β (13.1 kb, see ref. 37) contains the polyoma viral enhancer (fragment *Pvu*II (5,262)-*Bcl*I (5,021)) and two copies of the rabbit 4.5 kb chromosomal fragment including the rabbit β -globin gene³⁸. pSVBA34 (7.7 kb, see ref. 5) contains the SV40 enhancer (coordinates 113-270) linked to a fragment of the Ad-2 major late promoter (Ad-2MLP) (-34 to +33 with respect to the major late cap site) and to the SV40 large-T antigen coding region (coordinates 2,533-5,227). SE1 (3 kb) (see ref. 11) contains the SV40 enhancer (coordinates 113-270) cloned between the *Pvu*II and *Bam*HI sites of pBR322. E1AE (4.5 kb) contains the E1A enhancer sequences from position -500 to -145 (with respect to the E1A cap site) cloned between the *Eco*RI and the *Bam*HI sites of pBR322 (see ref. 11). None of the recombinants is drawn to scale. Solid boxes in pE1AG and p β (244+) β indicate β -globin exons.

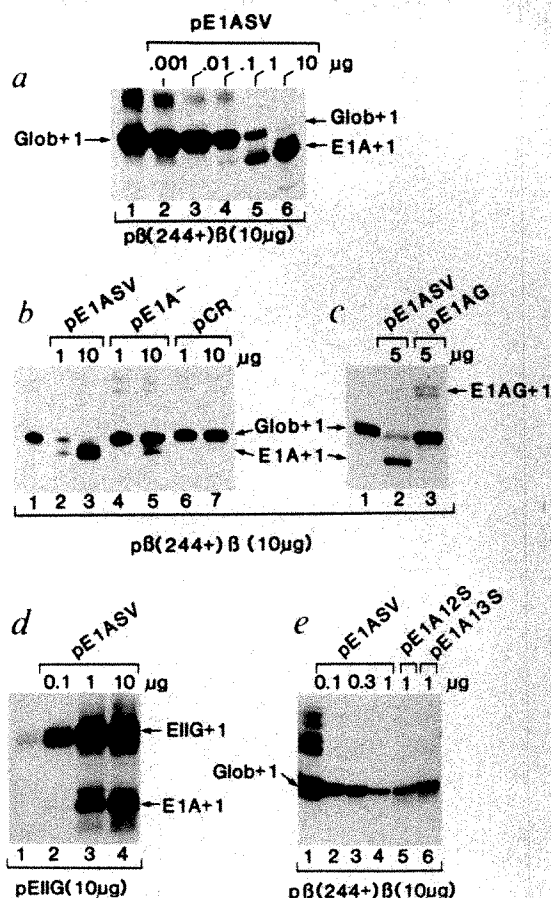


Fig. 2 Quantitative S_1 nuclease analysis of cytoplasmic RNA extracted from HeLa cells co-transfected with 10 μ g recombinant p β (244+) β and pE1ASV, pE1A⁻, pCR, pE1AG, pE1A12S or pE1A13S (a, b, c, e); d, 10 μ g recombinant pEIIG (see text and ref. 15) was co-transfected with increasing amounts of pE1ASV. The recombinants co-transfected and the amount of DNA transfected are indicated above the gel lanes. Transfection was carried out using the calcium phosphate procedure⁵ with the following modification: the cell monolayer was washed after 24 h and the cells collected 16 h later. The total amount of transfected DNA was kept constant at 20 μ g by the addition of pBR322. Cytoplasmic RNA was prepared⁵ and 10 μ g RNA hybridized with excess 5'-end labelled E1A and globin single-stranded probes^{5,12}, except in c, lane 3, and e, where the globin probe alone was used. The E1A probe is an *Eco*RI(-500)/*Sau*3A(+130) fragment and the β -globin probe a *Bst*NI fragment as described previously³². After S_1 nuclease treatment, the E1A-protected fragment (E1A+1), corresponding to RNA transcribed from pE1ASV or pE1A⁻, was 130 nucleotides in length. The globin-protected fragment (Glob+1), corresponding to RNA transcribed from p β (244+) β , was 134 nucleotides long, whereas RNA transcribed from pE1AG (E1AG+1) or pEIIG (EIIG+1) protected a 140-nucleotide long fragment. The decrease in RNA transcribed from the β -globin genes of p β (244+) β was estimated by densitometric scanning of autoradiograms corresponding to several experiments similar to a. Average decreases of 6-, 40- and 200-fold were obtained with 100 ng, 1 μ g and 10 μ g of co-transfected pE1ASV, respectively.

the E2a promoter and the rabbit β -globin coding sequence; see ref. 15). The results show clearly that the decrease in RNA transcribed from p β (244+) β and the activation of the E2a promoter occur in the same range of pE1ASV concentration (Fig. 2d).

The low molar ratio at which inhibition occurs suggests that it is not due to competition between the promoters of the two co-transfected recombinants. This hypothesis is supported by the results of co-transfection experiments with recombinant pE1A⁻, containing the E1A promoter region but not the E1A coding sequences (Figs 1 and 2b, lanes 4, 5), or recombinant pE1AG, in which the E1A coding region is replaced by the β -globin coding region (Figs 1 and 2c, lane 3). In neither case was there a decrease in the amount of RNA transcribed from the β -globin mRNA start site of p β (244+) β . In addition, *in vitro* nuclear run-on transcription experiments have shown previously that pE1A⁻ is more efficiently transcribed than pE1ASV (see ref. 15). Thus, competition between the promoters of the co-transfected recombinants cannot account for the decrease in transcription from the β -globin promoters of p β (244+) β . To exclude the possibility that the E1A coding region might contain a sequence trapping factors required for Py enhancer activity,

we constructed recombinant pCR, containing only the E1A coding sequences (Fig. 1). No decrease in β -globin RNA is observed when pCR is co-transfected with p β (244+) β (Fig. 2b, lanes 6, 7).

We next co-transfected pE1ASV with pSVBA34, a recombinant in which transcription from the +33 to -34 Ad-2MLP element is stimulated by the SV40 enhancer (Fig. 1). We found a decrease in RNA initiated from the Ad-2MLP, similar to those obtained previously with p β (244+) β (Fig. 4a, b, lanes 1, 2, data not shown). To examine the individual roles of the 12S and 13S mRNA products of the E1A transcription unit, we

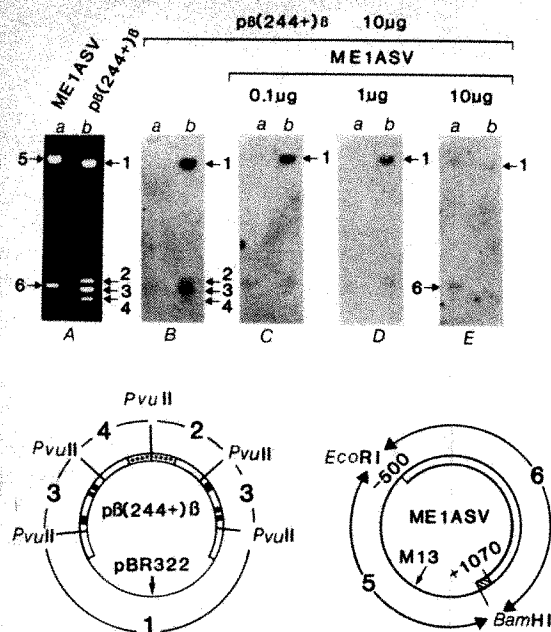


Fig. 3 Nuclear run-on transcription from nuclei of cells transfected with recombinants pβ(244+)β and ME1ASV. Lower part, ME1ASV was derived from pE1ASV by replacing the pBR322 sequences with Mpl1 sequences using the polylinker single *EcoRI* and *BamHI* sites. Upper part, restriction digests (2 μg DNA) of pβ(244+)β (lanes b) and ME1ASV (lanes a) were electrophoresed on a 1.5% agarose gel and blotted onto nitrocellulose filters as indicated. A, Ethidium bromide-stained agarose gel. Fragments 1-6 (see lower part) are 6.5, 1.8, 1.65, 1.5, 7.2, 1.7 kb in length, respectively. Fragment 3 contains the two β-globin genes of pβ(244+)β. B-E, Autoradiograms of the nitrocellulose blots after hybridization with labelled nuclear run-on RNAs prepared from HeLa cells transfected with pβ(244+)β alone (B) or with ME1ASV (as indicated).

Methods: pβ(244+)β was transfected into HeLa cells with increasing amounts of ME1ASV; nuclei were isolated, incubated *in vitro* (in the presence of heparin and ammonium sulphate) with ³²P-CTP and cold GTP, UTP and ATP and, after purification, RNA was hybridized to the above DNA fragments, as described in ref. 15.

co-transfected Py or SV40 enhancer-containing recombinants with cDNA-derived E1A recombinants producing either the 13S or the 12S mRNA (see ref. 15). Both E1A, 12S and 13S mRNA products can inhibit Py and SV40 enhancer activity (Fig. 2e, lanes 5, 6, and data not shown).

To rule out the possibility that the E1A products do not inhibit transcription activation by the enhancers, but rather interfere with the stability or the transport of the RNA produced, we performed run-on transcription experiments on isolated nuclei (Fig. 3). Co-transfection of 10 μg pβ(244+)β with as little as 0.1 μg of ME1ASV (a recombinant in which the pBR322 sequence of pE1ASV was replaced by M13 sequences) results in inhibition of transcription of the β-globin genes (Fig. 3B-E). As, in these nuclear run-on transcription experiments, the RNA synthesized corresponds only to elongation of RNA chains previously initiated *in vivo* (see ref. 15), we conclude that the E1A products repress the enhancer-mediated activation of β-globin gene transcription. In fact, a decrease in transcription from the other sequences of the recombinant pβ(244+)β is also observed (Fig. 3, fragments 1, 2, 4). The residual level of transcription obtained with pβ(244+)β when repression is maximal (10 μg ME1ASV, Fig. 3E), corresponds to the level obtained with a recombinant lacking the enhancer sequence (data not shown). This suggests strongly that the E1A products repress all enhancer-activated transcription in recombinant pβ(244+)β, including transcription initiated not only from the β-globin promoters, but also from pBR322 'substitute' promoter elements³.

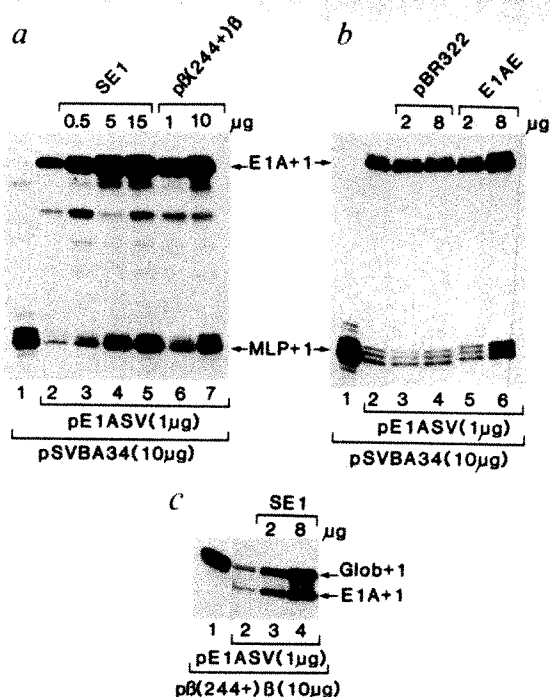


Fig. 4 Co-transfection with recombinants containing the Py virus or SV40 enhancers relieves repression of enhancer-mediated transcription by the E1A products. The experimental procedure is described in Fig. 2 legend. a, b, Lanes 1, 10 μg pSVBA34 was transfected alone. a, b, Remaining lanes, pSVBA34 and pE1ASV were transfected in the amounts indicated. In a, lanes 3-7, b, lanes 3-6, a third recombinant was co-transfected. The nature of this recombinant and the amount of DNA transfected are indicated above the gel lanes. c, 10 μg pβ(244+)β was transfected either alone (lane 1) or with 1 μg of pE1ASV (lanes 2-4). A third recombinant, SE1, was co-transfected in lanes 3 and 4 as indicated. RNA transcribed from pE1ASV (E1A+1) and pβ(244+)β was analysed by quantitative S₁ nuclease mapping as indicated in Fig. 2 legend. The probe used for analysing RNA initiated from pSVBA34 at the Ad-2MLP cap site was the 5' end labelled single-stranded *HindIII-XhoI* fragment (see ref. 5) giving a protected fragment of 93 nucleotides (MLP+1).

Involvement of *trans*-acting factor(s)

To investigate whether repression of the enhancer-mediated stimulation of transcription by the E1A products is a *trans*-acting process involving an interaction with the enhancer, we attempted to remove the repression by co-transfecting increasing amounts of a third recombinant containing only a purified enhancer element cloned in pBR322. Increasing amounts of SE1 (a recombinant in which the SV40 enhancer is cloned in pBR322, see Fig. 1) in co-transfections where transcription of pSVBA34 (Fig. 4a, lanes 2-5) or pβ(244+)β (Fig. 4c, lanes 2-4) is repressed by pE1ASV, derepresses transcription initiated either from the Ad-2MLP (MLP+1) in Fig. 4a, compare lanes 1-5) or from the β-globin promoter (Glob+1) in Fig. 4c, compare lanes 1-4). The molar ratios of SE1 : pSVBA34 are 0.13, 1.3 and 3.9 in lanes 3-5 of Fig. 4a, whereas those of SE1 to pβ(244+)β are 0.9 and 3.6 in lanes 3 and 4 of Fig. 4c. Similarly, the addition of the Py enhancer in the form of the recombinant pβ(244+)β to a transfection also containing pSVBA34 or pE1ASV relieves the repression of transcription from the Ad-2MLP (Fig. 4a, compare lanes 1, 2, 6 and 7; the molar ratios of pβ(244+)β to pSVBA34 are 0.17 and 1.7 in lanes 6 and 7, respectively). In contrast, no 'derepression' is observed when pBR322 is transfected with pSVBA34 and pE1ASV (Fig. 4b, lanes 3, 4). We conclude from these results that repression of enhancer activity by the E1A products may involve an interaction between the enhancer and either the E1A products or some cellular factor(s) induced by the E1A products, or a combination of both.

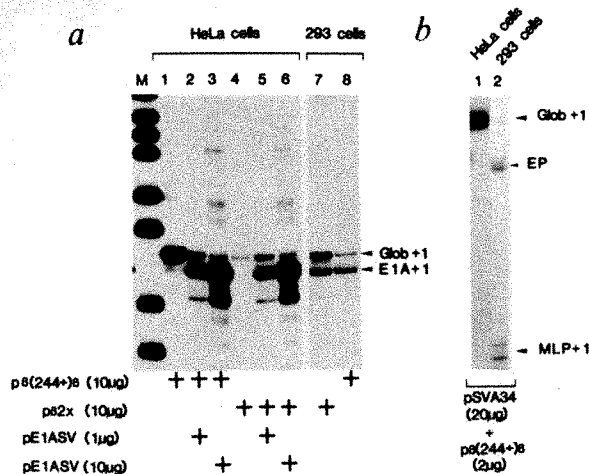


Fig. 5 Quantitative S_1 nuclease analysis of RNA synthesized in HeLa or 293 cells transfected with pβ(244+)β, pSVA34, pβ2X and pE1ASV as indicated. pSVA34 (ref. 5) is similar to pSVBA34 (Fig. 1), except that it does not contain the SV40 enhancer. pβ2X is similar to pβ(244+)β (Fig. 1) except that it does not contain the polyoma virus enhancer (see ref. 37). The probes used for S_1 nuclease mapping of RNA initiated from the Ad-2MLP (MLP+1), the β-globin promoter (Glob+1) or the E1A promoter (E1A+1) are described in Figs 2 and 4 legends. Hybridization reactions contained either 10 μg cytoplasmic RNA from HeLa cells or 20 μg cytoplasmic RNA from 293 cells. Other experimental procedures are described in Fig. 2 legend. EP, end point of homology between transcripts initiating in pBR322 and the Ad-2 major late probe.

In the derepression experiments (Fig. 4a, c), the amount of RNA initiated at the E1A mRNA start site (E1A+1) increases in parallel with that of RNA initiated from either the Ad-2MLP (Fig. 4a, lanes 3-7) or the β-globin promoter (Fig. 4c, lanes 3, 4). These results suggest that E1A products can also repress transcription stimulated by the E1A enhancer(s)²³⁻²⁵. This hypothesis is supported by the increase of RNA initiated from the Ad-2MLP (MLP+1) and E1A promoter (E1A+1) when a recombinant containing the E1A enhancer region inserted in pBR322 (E1AE, see Fig. 1) is transfected with pSVA34 and pE1ASV (Fig. 4b, compare lanes 1, 2, 5 and 6).

E1A stimulation of enhancer-less promoters

E1A products expressed either permanently in the 293 cells transformed by the adenovirus-5 E1 genes, or transiently in HeLa cells after transfection with the Ad-2 E1A transcription unit, can stimulate transcription from a variety of genes transfected into these cells. Strikingly, transcription can be stimulated in these conditions not only from the other adenovirus early genes^{15,22,25}, but also from recombinants containing cellular transcription units^{20-22,26}. The E1A products thus appear to act as activators of transcription from RNA polymerase class B (II) promoters, perhaps by interacting with the TATA box region or the factor(s) recognizing it²⁰. Interestingly, the SV40 enhancer has no effect on rabbit β-globin gene expression in 293 cells²¹ or in HeLa cells²² co-transfected with a recombinant expressing the E1A products. In the latter case, the presence of SV40 enhancer even results in a two- to threefold decrease in RNA transcribed from the rabbit β-globin gene.

There is no real discrepancy between our results and those published previously (Fig. 5a; refs 20-22). E1A products clearly stimulate transcription from the rabbit β-globin gene promoter in the absence of an enhancer (Fig. 5; compare lanes 4-6, where pβ2X, a derivative of pβ(244+)β that does not contain an enhancer, is transfected either alone or with increasing amounts of pE1ASV). In identical transfection conditions, co-transfection of the Py enhancer-containing rabbit β-globin recombinant pβ(244+)β and the plasmid expressing the E1A products (pE1ASV) strikingly decreases transcription from the β-globin

promoter (Fig. 5, lanes 1-3). At the higher pE1ASV concentration, the amounts of β-globin RNA synthesized from the enhancer-containing pβ(244+)β recombinant (Fig. 5, lane 3) and the enhancer-less pβ2X recombinant (Fig. 5, lane 6) are very similar. This suggests that when a recombinant expressing the E1A products (pE1ASV) is co-transfected with an enhancer-containing β-globin gene recombinant pβ(244+)β, the E1A products abolish the activity of the enhancer, but are still capable of stimulating transcription from the β-globin promoter. These observations may explain also previous conclusions that an enhancer does not increase the amount of RNA transcribed from the rabbit β-globin gene in HeLa cells co-transfected with a plasmid expressing the E1A products²².

It is also clear that in 293 cells, the rabbit β-globin gene is not expressed at a higher level in a recombinant containing the Py enhancer (Fig. 5a, lane 8) than in a recombinant lacking it (pβ2X; Fig. 5a, lane 7). (The amount of β-globin RNA in lane 8 should be corrected for a lower content in E1A RNA when compared with the amount of β-globin RNA signal in lane 7.) The stimulatory effect of the Py enhancer is cancelled in 293 cells, whereas the activity of the β-globin promoter of the enhancer-less recombinant pβ2X is still stimulated by the E1A products synthesized in these cells (Fig. 4; compare lanes 1 and 4 with lanes 7 and 8). However, as there are no control cells for 293 cells, these results cannot be directly compared with those obtained with pE1ASV-transfected HeLa cells. Similar results are obtained when the Py enhancer-containing recombinant pβ(244+)β and the Ad-2MLP enhancer-less recombinant pSVA34 (pSVBA34 lacking the SV40 enhancer; Fig. 1) are co-transfected into HeLa or 293 cells (Fig. 5b). The level of expression of these two recombinants in HeLa and 293 cells cannot be compared directly because a co-transfected reference plasmid cannot be used. However, in agreement with the above conclusion, there is a clear inversion in the relative level of expression of these recombinants in HeLa and 293 cells. Relative to the enhancer-containing recombinant pβ(244+)β, the enhancer-less pSVA34 plasmid is expressed more efficiently in 293 cells (Fig. 5b, lane 2), whereas, relative to pSVA34, pβ(244+)β is better transcribed in HeLa cells (Fig. 5b, lane 1).

Conclusion

The results described here demonstrate that the stimulation of transcription mediated by enhancers can be negatively regulated. Because it is possible to compete the negative effect of the E1A products by co-transfecting recombinants containing only an enhancer element, and also because repression of the enhancer activity does not interfere with the ability of the E1A products to stimulate transcription from the β-globin promoter, the enhancer itself is probably the target for the negative regulation. Future studies will determine whether the E1A products themselves, or a cellular product(s) induced by them, or some combination of the two, interact with the enhancer sequence and/or with other enhancer factors. Using an extensive set of SV40 enhancer mutants (M. Zenke, T. Grundström, H. Matthes, M. Wintzerith and P.C., manuscript in preparation), we will determine which enhancer sequences are involved in E1A repression. In this respect, the only sequence feature which is apparently shared by the SV40, Py virus and Ad-2 enhancers is the core sequence^{23,27}. *In vivo* studies have clearly shown that the SV40 enhancer can generate an altered chromatin structure over its own sequence²⁸. Recent *in vitro* studies demonstrate that a transcriptional factor(s) interacts specifically with the SV40 enhancer⁹⁻¹¹. We are now investigating whether the E1A products interfere with either of these enhancer functions.

At present it is not known whether the E1A products inhibit the activity of the E1A enhancer during lytic infection or whether the inhibition could be observed only with an isolated E1A transcription unit. However, inhibition of the E1A enhancer activity by the E1A products provides a means by which the expression of the E1A transcription unit can be autoregulated during lytic infection. Indeed, it has been reported that E1A transcriptional activity is highest during the early phase of

infection^{13,14}. On the other hand, there is evidence that the E1A products stimulate transcription of the E1A transcription unit early in adenovirus infection^{29,30}. It appears, therefore, that the E1A products can act as both negative and positive regulators of their own transcription units during lytic infection, and as positive regulators of all other viral transcription units (see refs 13-15). Note that the SV40 early gene product, the large-T antigen, also regulates its own synthesis, but by a more 'classical' repression mechanism that does not involve an interaction with the SV40 enhancer³¹. In addition, like the E1A products, the large-T antigen is a pleiotropic protein also activating transcription, as it has been reported recently that it stimulates the activity of the SV40 late promoter³². It is not known whether inhibition of cellular enhancers by the E1A products occurs during productive infection or during cell transformation by adenoviruses. As yet, the expression of only one defined gene has been shown clearly to be repressed by E1A products. The expression of a class I major histocompatibility complex (MHC) gene is repressed in rat cells transformed by the E1A transcription unit of the highly oncogenic adenovirus-12 (Ad-12), but not by that of the weakly oncogenic adenovirus-5 (ref. 19). Since the E1A products of Ad-12 also inhibit activation of transcription by SV40 and Py enhancers (our unpublished observations), we are now investigating the possibility that this class I MHC gene contains an enhancer whose activity can be repressed by the Ad-12 E1A products.

Received 1 August; accepted 9 October 1984.

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Gene expression in eukaryotic cells may be controlled by regulatory factors repressing enhancer activity. This has not yet been reported. However, the Py enhancer is not active in undifferentiated embryonal carcinoma cells, although mutations in the region containing the Py enhancer restore its activity in these cells³³⁻³⁵. It has been suggested that embryonal carcinoma cells express a protein with properties similar to those of the E1A products³⁶. This protein may repress the activity of the Py enhancer. Thus, in addition to demonstrating that the adenovirus E1A products have both negative and positive regulatory functions, our study raises the possibility that cellular enhancers are regulated not only positively as suggested previously^{1,2,4,6-8}, but also negatively, which would significantly increase the combinatorial possibilities for the control of gene expression at the transcriptional level in eukaryotes.

We thank P. Sassone-Corsi, T. Leff and T. Reudelhuber for critical reading of the manuscript; W. Schaffner, P. Sassone-Corsi, C. Goding and C. Kédinger for gifts of recombinants; B. Boulay, A. Landmann, B. Chambon, C. Aron and C. Werlé for illustration and preparation of the manuscript and B. Augsburg and M. Acker for cell culture. E.B. receives a fellowship from Université Louis Pasteur Strasbourg. This work was supported by CNRS (ATP 3582), INSERM (PRC 124026), Ministère de l'Industrie et de la Recherche (82VI283), Fondation pour la Recherche Médicale and Association pour le Développement de la Recherche sur le Cancer.

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A bacterial repressor protein or a yeast transcriptional terminator can block upstream activation of a yeast gene

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A bacterial repressor protein blocks transcription of a gene in yeast when its operator is placed in the promoter between the upstream activator sequence and the transcription start point. Putative transcription terminators from yeast installed in the same region of the promoter have a similar effect.

PROMOTERS recognized by RNA polymerase II of the yeast *Saccharomyces cerevisiae* contain two essential elements: a stretch of DNA containing a sequence homologous to TATA, and an upstream activator sequence (UAS)¹. For example, synthesis of *GAL1* mRNA, which begins 80 nucleotides downstream of the TATA region of the *GAL1* promoter, depends on a sequence called UAS_G located about 250 nucleotides upstream

of the TATA region²⁴ (see Fig. 1). UAS_G function has been localized to a 75-nucleotide sequence², and, more recently, to a 15-base pair (bp) sequence with approximate 2-fold rotational symmetry (E. Giniger *et al.*, in preparation). The *GAL4* gene product is thought to bind to this sequence in the presence of galactose to activate transcription downstream. When it is placed in front of the TATA region of *CYC1*, UAS_G activates transcrip-

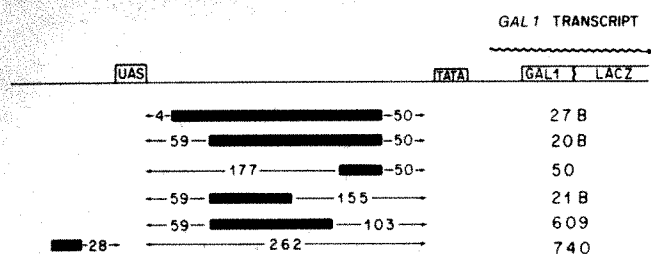


Fig. 1 Derivatives of the *GAL1* promoter. All carried a *XhoI* site into which *lexA* operators were inserted. Distance from the *XhoI* site to the TATA region and to the upstream activator sequence for various promoter derivatives is given in nucleotides. The *UAS_G* region contains two homologous 15-nucleotide regions of approximate 2-fold rotational symmetry separated by three nucleotides (one of which is sufficient for *UAS_G* function; E. Giniger *et al.*, in preparation). When defined in this way, there are 262 nucleotides between *UAS_G* and the first base of the TATA sequence. Numbering in *GAL1* derivatives is from the centre of the *XhoI* recognition sequence to the closest base in the upstream activator sequence, and to the first T of the *GAL1* TATA sequence. The mature *GAL1* transcript begins 85 nucleotides downstream of this T¹, and the *GAL1* coding sequence begins 60 nucleotides farther downstream^{2,4}. The extent of promoter deleted in each derivative is shown by a dark bar. 36 nucleotides are deleted in 740. Plasmids containing sites for *lexA* operator insertion were made by combining members of two sets of deletions². 609 and 740 were constructed as described in ref. 2. 20B and 27B are described in ref. 2. 50 and 21B were gifts of M. S. Lamphier.

tion from the hybrid promoter and renders this transcription inducible by galactose⁵. The distance between *UAS_G* and a TATA region is not critical, and the effect of *UAS_G* is still measurable even when it is located 500 nucleotides upstream of a TATA region (R. Yocum, personal communication). In these respects, *UAS_G* shares properties with the transcriptional enhancer sequences found in higher eukaryotes.

We describe two different ways to block *GAL1* promoter function by interposing two different kinds of genetic elements between its upstream activator sequence and its TATA region.

We constructed a strain of yeast that synthesizes *lexA* protein, a repressor protein from *Escherichia coli*. We also constructed variants of the yeast *GAL1* promoter that carried *lexA* operators at different positions within the promoter. We found that those *GAL1* promoters carrying a *lexA* operator between the upstream activator sequence and the TATA region were repressed by *lexA* protein.

We next installed sequences located downstream of the coding regions of the *CYC1* and *ADH1* genes between the *GAL1* upstream activator sequence and its TATA region. These sequences diminished *GAL1* transcription. A fragment of DNA containing a mutation interfering with proper *CYC1* termination and differing from the terminator fragment by a single base pair, was less able to decrease *GAL1* transcription.

LexA protein represses transcription

LexA protein is a repressor of many of the genes induced in *E. coli* as a consequence of DNA damage⁶⁻⁹. A plasmid directing the synthesis of LexA protein in yeast was constructed (Fig. 2). Use of anti-LexA antiserum allowed us to detect LexA protein in extracts of yeast carrying this plasmid. We estimate that the LexA protein comprises 0.005–0.025% of the total protein when yeast containing this plasmid are grown on galactose-containing medium. We inserted synthetic *lexA* operators into derivatives of the *GAL1* promoter (described in Fig. 1 legend) and chose a *lexA* operator sequence based on analysis of the sequence of many LexA protein-binding sites and on some of their interactions with the protein. Gel analysis of DNA–protein complexes¹⁰ (K. Chapman and R.B., unpublished data) revealed that the synthetic 22-nucleotide operator bound LexA protein at least as tightly as any single naturally occurring operator thus far

Table 1 LexA protein repression of *GAL1* promoter with *lexA* operators

	Control plasmid	<i>lexA</i> plasmid
20B	5,000	5,000
+1 op	2,500	350
+2 op	2,500	250
27B	1,800	1,800
+1 op	1,000	150
50	5,000	5,000
+1 op	2,500	600
+2 op	2,400	500
21B	5,000	5,000
+1 op	2,500	400
+2 op	2,300	250
609	5,000	5,000
+1 op	3,100	400
740	5,000	5,000
+1 op	5,000	5,000

Yeast was transformed with two types of plasmids having two different selectable markers. One plasmid directed the synthesis of LexA protein and carried the *LEU2* marker. Another plasmid contained a derivative of the yeast *GAL1* promoter with a *lexA* operator site and carried the *URA3* marker. Transformed yeast were isolated and propagated on medium that selected for cells bearing both plasmids. The amount of transcription from the *GAL1* promoter was measured after cultures of doubly transformed cells had been grown on galactose-containing medium for at least five generations. The derivatives of the *GAL1* promoter in which *lexA* operators were installed are shown in Fig. 1. Synthetic *lexA* operators with the sequence

TCGAGTACTGTATGTACATACAGTAC

CATGACATACATGTATGTATGATGAGCT

were inserted directly into *XhoI*-cut *GAL1* promoter derivatives (shown in Fig. 1) using standard plasmid construction techniques¹⁹. To construct a given doubly transformed strain, DNA from one of these plasmids and DNA from either pAAH5 (control plasmid) or pRB500 (which directed the synthesis of LexA protein) was used to transform strain DBY 745 to *URA⁺* and to *LEU⁺*²⁰. The amount of *GAL1* transcription in a given strain was estimated from indicator plates²⁸. The numbers in this table and in the tables below refer to units of β -galactosidase measured in liquid assays as described in ref. 3. Each strain was assayed in triplicate on at least five different occasions. Normally the level of β -galactosidase in a given transformed strain varied by no more than 20% on the day of the assay. Assay-to-assay variation of the ratio of galactosidase activity from any two different strains was usually less than 10%. Measured units were averaged and rounded to the nearest 50 units. op, Operator.

Table 2 Effect of LexA protein on transcription of an operator containing *GAL1* promoter integrated into the chromosome

Control plasmid	2,000
<i>lexA</i> plasmid	200

Plasmid 118 (20B+2*lexA* operators) was partially digested with *EcoRI*, ligated, and the ligation mixture cut with *XbaI* to linearize plasmids that retained the *EcoRI* fragment from 118 containing the 2 μ origin. The ligation mixture was used to transform *E. coli* strain JM101²¹, which lacks a functional *lacZ* gene. 427, a derivative of 118 that lacks the *EcoRI* fragment containing the replication origin, was isolated from a *lacZ⁺*, Amp^R colony. 427 was cut in its *URA3* gene with *ApaI* and lithium acetate-treated DBY745 was transformed (see Table 1 legend) with the cut plasmid DNA. Six *URA* transformants arising from this construction were checked to see that the *URA* marker was not lost after growth for 40 generations on nonselective medium, and that *GAL1* promoter transcription in these strains was normally inducible if the yeast were grown on medium containing galactose. These six strains were made competent with lithium acetate, then transformed to *LEU⁺* with a plasmid directing the synthesis of LexA protein, pRB500, or with its parent plasmid, pAAH5. β -Galactosidase in these strains was assayed, in liquid and on indicator plates, as described in Table 1. There was no variation in *GAL1* transcription among the six strains when grown on galactose, glucose, and galactose with *GAL1* transcription repressed by LexA protein.



Fig. 2 *E. coli* LexA protein manufactured in yeast. Lane 1, 1 μ g of purified LexA protein⁹. About 10% of this LexA protein preparation consisted of the amino- and carboxy-terminal autolytic fragments²³. The carboxy-terminal fragment is visible on the gel. Lane 2, extract from DBY745/pAAH5. Lane 3, extract of DBY745/pRB500. Lane 4, extract of DBY745/pRB500. Lane 5, extract of DBY745/pAAH5, spiked with 50 ng purified LexA protein.

Methods: pRB500, a plasmid which directs the synthesis of LexA protein, was made by inserting a 1,000-nucleotide *HindIII*-*HindIII* piece from pRB480 (unpublished data), which contained DNA from 62 bases upstream to ~200 nucleotides downstream of *lexA*, into the *HindIII* site of pAAH5. pAAH5 is a plasmid which carries the *LEU2* gene, a 2 μ origin of replication, and several hundred nucleotides of DNA from around the *ADH1* (*ADCI*) promoter. The *ADH1* fragment ends 11 nucleotides upstream of the ATG that would begin the *ADH1* structural gene. At this site a *HindIII* linker has been inserted. pAAH5 was constructed by Gustav Ammerer. Yeast strain DBY745 (α *adel-100 leu2-3 leu2-112 ura3-52*) was transformed with pAAH5 or pRB500, using the lithium acetate technique (M. Rose, personal communication). To produce each extract, yeast were grown in glucose minimal medium without leucine¹⁸ to a density of 2×10^7 ml⁻¹. 2 ml of this culture were concentrated by centrifugation, resuspended in 0.2 ml Laemmli sample buffer²⁴, and lysed by grinding with glass beads²⁵. 0.05 ml of each extract was run on a 12% polyacrylamide/SDS gel²⁴. Proteins in the gel were transferred electrophoretically to a nitrocellulose membrane; the membrane was probed with anti-LexA protein antibody followed by ¹²⁵I-protein A from *Staphylococcus aureus*¹³. The membrane was washed and exposed to XAR-5 film for 24 h. In other experiments, protein gels of lysates of yeast that carry this plasmid were stained with Coomassie brilliant blue and revealed a faint new band whose mobility was the same as that of pure LexA protein (not shown). LexA protein was also observed in these strains after cellular proteins were labelled with ³⁵S-methionine and immunoprecipitated with anti-LexA antiserum (not shown). From immunoprecipitation experiments and from stained gels, we estimated that LexA protein constituted between 0.02% and 0.1% of the total soluble protein in cells transformed with this plasmid grown in glucose. As the *ADH1* promoter is about five times less active when cells are grown in galactose as when cells are grown in glucose (refs 26, 27 and our unpublished results), we suspect that there is one-fifth this amount of LexA protein in galactose-grown yeast.

examined^{7,9}, with an apparent dissociation constant (K_d) of $<10^{-9}$ M. The *GAL1* promoter derivatives were carried on plasmids that supplied a replicator and a selectable marker. The *GAL1* structural gene was fused in each case to a *lacZ* gene so that the β -galactosidase level in a plasmid-carrying cell was a measure of the *GAL1* transcription.

Derivatives of the *GAL1* promoter containing *lexA* operators between the upstream activator sequence and the TATA region (derivatives 27B, 20B, 50, 21B and 609; Fig. 1) were repressed

Table 3 Putative transcriptional terminators, installed between the *GAL1* upstream activator sequence and its TATA region, decrease *GAL1* transcription

20B	5,000
+ <i>cycl-512</i>	2,500
+ <i>cycl-512-F</i>	800
+ <i>ADH1</i> terminator	50
+ 82-nucleotide polylinker	5,000
+ 234-nucleotide pBR322 <i>HaeIII</i> fragment	5,000

Plasmids carrying *cycl-512* and *cycl-512-F* *RsaI*. pAAH5 was cut with *HindIII* and *SphI*. Fragments containing putative transcriptional terminators, the 182-nucleotide *EcoRV*-*Rsa* fragment, and the 324-nucleotide *HindIII*-*SphI* fragment, were isolated and inserted into the *StuI* site of pRB486 (unpublished construction). The unique *StuI* site in this plasmid arises from the juxtaposition of two *XhoI* linkers. Derivatives of pRB486 that carried each mutation were cut with *XhoI* and the *XhoI*-ended terminator fragments inserted into the *XhoI* site of 20B. Compared with the wild-type promoter, the *GAL1* promoter derivative carried on 20B has 153 nucleotides missing. Derivatives of 20B that carried these terminator fragments in the wild-type orientation were used to transform DBY745. A similar strategy was used to insert into 20B an 82-nucleotide *HindIII*-*HaeIII* polylinker fragment from plasmid 701 (unpublished), a derivative of puc19²¹. 20B-G is a derivative of 20B that contains, in its *XhoI* site, a 234-nucleotide *HaeIII* fragment from pBR322 (ref. 22). This plasmid was constructed by Jun Ma. Yeast bearing these plasmids were grown on galactose and assayed on indicator plates and in liquid as described in Table 1 legend.

4-10-fold by LexA protein (Table 1). All *GAL1* promoter derivatives containing two *lexA* operators were repressed more efficiently than corresponding derivatives that contained only a single operator. No repression was observed when the operator was installed upstream of the *UAS_G* (in *GAL1*, derivative 740; see Fig. 1). In the absence of LexA protein, *lexA* operators at certain sites in the *GAL1* promoter slightly diminished transcription (Table 1). This effect may be related to the slight homology between the *lexA* operator and sequences implicated in yeast transcription termination (unpublished data).

We sought to confirm that the apparent *lexA*-mediated repression of operator-containing *GAL1* promoters was not caused by variation in plasmid copy number. Accordingly, we constructed nonreplicating derivatives of two *lexA*-repressible *GAL1* plasmids and directed their integration into a yeast chromosome²⁹. We examined six different integrants of each plasmid and found that repression by LexA protein was as efficient, but no more efficient, than the repression observed when the operator-containing *GAL1* promoter was present in many copies on a plasmid. Repression of one such integrated promoter is shown in Table 2.

Upstream terminators decrease transcription

Sequences downstream of certain yeast genes have been identified tentatively as sites where transcription terminates; it is possible, although less likely, that these sequences are signals which cause a longer transcript to be processed¹¹⁻¹². Putative transcription terminators are found downstream of the coding sequences of the *CYC1* and *ADH1* (*ADCI*) genes (see Fig. 3 legend). We inserted fragments of DNA carrying these putative terminators or mutant derivatives of them between the *GAL1* upstream activator sequence and its TATA region. We placed the terminator fragments in a deletion derivative of the *GAL1* promoter to ensure that the distance between the upstream activator sequence and the TATA region in the terminator-containing construction was approximately that found in the wild-type promoter. One fragment was taken from the 3' end of a *CYC1* gene (the allele *cycl-512-F*) in which the correct messenger RNA (mRNA) is formed. Another fragment differs by a single base pair and was taken from a mutant (*cycl-512*) in which the correct 3' end of the mRNA is only formed inefficiently, presumably because termination is rendered defective by the changed base pair (refs 11, 12 and K. Zaret, M. Hampsey and F. Sherman, personal communication). In addi-

tional experiments, we used a fragment that carried the putative transcriptional terminator from the 3' end of the *ADHI* gene¹².

The fragments derived from the two *CYC1* alleles differed in their ability to depress *GAL1* transcription. The fragment carrying the putative transcriptional terminator (*cyc1-512-F*) decreased *GAL1* transcription by a factor of six (Table 3). In contrast, the fragment derived from the termination-deficient mutant (*cyc1-512*) decreased transcription by a factor of two. The fragment derived from *ADHI* showed a larger effect. Installation of this piece of DNA decreased *GAL1* transcription by 100-fold. Control pieces of DNA from pBR322 and pUC19, installed in the same deletion, had no effect on *GAL1* transcription (Table 3). If the *CYC1* terminator is inserted between the *CYC1* upstream activator sequence and its TATA region, the ability of a fragment to depress transcription is correlated with its ability to function as a transcriptional terminator (B. Osborne and L. Guarante, personal communication).

Discussion

Our experiments provide genetic evidence that a bacterial repressor protein manufactured in the yeast cytoplasm can enter the yeast nucleus, recognize its operator and repress transcription from a yeast promoter. Immunofluorescence experiments (performed in collaboration with P. Silver) show that LexA protein is localized to the nucleus in a minority of cells in a population that produces it (data not shown). In most members of the population, fluorescence is uniformly spread throughout the cytoplasm and we conclude that LexA protein has passively entered the nucleus. This behaviour is in contrast to the strictly nuclear localization of regulatory proteins native to yeast, which are thought to have a distinctive stretch of amino acids causing them to enter and remain in the nucleus (refs 13, 14, R. Moreland and L. Hereford, personal communication). Uniform cellular distribution of LexA protein should be sufficient to repress operator-containing *GAL1* promoters. If LexA protein constitutes 0.025% of the total protein in cells grown on galactose, and if it is evenly distributed throughout the cell, there should be about 750 LexA molecules in the nucleus. As the volumes of *E. coli* and a yeast nucleus are about the same, nuclear LexA protein concentration is probably equivalent to the concentration found in a single *E. coli*, which contains 100–1,000 molecules of LexA protein (unpublished data). We have shown here that the affinity of the synthetic operator for LexA protein is at least as high as that found for naturally occurring *lexA* operators. Therefore, 100–1,000 molecules of LexA per nucleus should be sufficient to occupy the operator¹⁵.

Consider two possible mechanisms by which LexA protein might repress *GAL1* transcription. First, LexA protein could repress *GAL1* transcription because its binding site is close to or overlaps the binding site of some protein necessary for transcription. Although some sites of operator insertion are located far from either the upstream activator sequence or the TATA region, all sites are at least moderately close (within 59 nucleotides) to one region or the other. Therefore, we cannot exclude this possibility. Note, however, that LexA protein has no effect at a site 28 nucleotides upstream of the upstream activator sequence, so proximity of an operator to the upstream activator sequence is not sufficient to produce a repressible promoter.

Second, the observed repression may be caused by LexA protein repressing *GAL1* transcription by blocking transmission of some stimulus (for example, RNA polymerase or some component of the transcription apparatus) from the upstream activator sequence to the TATA region. These two mechanisms are not mutually exclusive; depending on the location of the *lexA* operators within the *GAL1* promoter, one or both of them may exist.

Our results with transcription terminators reported here might be regarded as evidence in support of the second proposed mechanism. Transcription between the upstream activator sequence and the TATA region, however, has never been observed. It is possible that RNA polymerase makes an unstable transcript between the upstream activator sequence and the observed transcription start point, or that RNA polymerase or some other component of the transcription apparatus moves through this region in some other manner sensitive to the effects of transcription terminators. But we cannot exclude the possibility that a protein binds to the functional terminator but not to the mutant terminator, and that the bound protein interferes with transcription in the same way as LexA protein.

There is evidence that other yeast genes can be repressed by negative regulatory factors bound between the upstream activator sequence and the TATA region. For example, it is likely that *MATA1* transcription is repressed in diploid yeast by binding of a negative regulatory factor (which is dependent on the expression of *MATA1* and *MAT α 2*) to a site between the *MATA1* upstream activator sequence and its TATA region¹⁶. *STE6* transcription is repressed by the *MAT α 2* product, which binds to a specific site in the *STE6* promoter, probably located between the upstream activator sequence and the TATA region (A. Johnson, K. Wilson and I. Herskowitz, personal communication). The repression of *MATA1* and *STE6* is at least 50 times greater than the 10-fold repression we observe for *lexA* operator-containing derivatives of the *GAL1* promoter. We do not understand why these yeast promoters are repressed so efficiently relative to the *lexA* operator-containing *GAL1* promoter.

Further studies may use well-characterized prokaryotic regulatory proteins to control expression of other yeast genes and genes in higher eukaryotes. It will be of interest to see whether repressor proteins can block the activation of genes by enhancer sequences¹⁷ found in higher eukaryotes. Repressor proteins may also be useful in studying the mechanism of negative regulation in yeast in those cases where negative control is exerted at a distance, such as the repression of silent genes of the yeast mating type locus by the *MAR/SIR* gene products¹⁸.

We thank Susan Varnum for technical assistance; Ken Zaret, Michael Hampsey and Fred Sherman for *CYC1* mutations and discussion of their unpublished results; Pamela Silver, Rog Yocum, Jack Szostak, Mark Rose, Robin Wharton, Robert West and Liam Keegan for helpful discussions; Robert West and Marc S. Lamphier for strains; John Douhan III for anti-LexA antibody; Alexander Johnson, Katherine Wilson, Ira Herskowitz, Brian Osborne and Leonard Guarente for communicating unpublished results; Ann Hochschild, Kai Zinn, Anne Ephrussi and Edward Giniger for comments on the manuscript. This work was supported by NIH grants GM29988 and GM32308.

Received 5 September; accepted 22 October 1984.

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Primary structure and genomic organization of the histidine-rich protein of the malaria parasite *Plasmodium lophurae*

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The nucleotide sequence of a complete genomic clone for the histidine-rich protein of Plasmodium lophurae has been determined. The deduced amino acid sequence of the mature protein shows numerous tandemly repeated units preceded by a signal and a pro peptide. The gene is interrupted by an intron with a separate exon coding for the signal peptide. The signal peptide-encoding exon detects multiple cross-hybridizing sequences in the parasite genome.

THE protozoan parasite *Plasmodium lophurae* causes malaria in birds, invading host erythrocytes via a mechanism which involves specialized intracellular parasite organelles and surface receptors on both the parasite and the erythrocyte¹⁻⁴. Recognition and binding of the two cells is followed by complete engulfment of the protozoan cell by the erythrocyte, whereupon the parasite undergoes several rounds of asexual divisions. Mature daughter cells escape from their intraerythrocytic confinement to begin a new round of erythrocyte invasion.

In the intraerythrocytic stages of development of *P. lophurae* in ducks, there is synthesis of a major protein that accumulates to comprise at least 50% of the cellular mass. This protein, a basic polypeptide of relative molecular mass (M_r) 45,000 comprising 73% histidine, is located in a membrane-bounded compartment that forms part of the specialized parasite organelles implicated in erythrocyte invasion⁵. The function of the protein is unknown. In one series of experiments, antibodies to this protein were found to be protective⁶, but other investigators have been unable to reproduce these results^{7,8}.

We have shown that the early biosynthetic forms of the histidine-rich protein resemble those of secretory proteins⁹. Translation of parasite mRNA in a cell-free wheat-germ translation system yielded a larger precursor that was translocated into dog pancreas microsomal membrane vesicles. The segregated form was larger than the mature protein and contained Asn-linked oligosaccharide⁹. These data suggested that the histidine-rich protein is synthesized as the prepro-protein containing two transient sequences, a pre-sequence that functions as a signal sequence for translocation across the rough endoplasmic reticulum, and a glycosylated pro-sequence of unknown function.

We have now isolated a genomic clone that contains the entire histidine-rich protein gene and have determined its DNA sequence. The gene is encoded in two exons, separating the signal peptide-encoding sequence from the pro-sequence, confirming that synthesis of the protein occurs via the prepro-protein. Oligonucleotide probes synthesized to the signal peptide-encoding exon reveal multiple homologous DNA sequences in the *P. lophurae* genome. The sequence of mature protein is arranged in numerous tandem repeats with up to nine histidine residues in a row, similar to other *Plasmodium* proteins for which sequence data have so far been reported^{10,11,36-39}.

Genomic clone isolation

A partial cDNA clone, obtained by screening a *P. lophurae* cDNA library with a synthetic oligonucleotide encoding a poly-histidine sequence (ref. 12 and R. Feder *et al.*, in preparation), was used to determine the genomic organization of the histidine-rich protein. *P. lophurae* DNA digested with *Hind*III (Fig. 1a, lane 3) or *Eco*RI (Fig. 1a, lane 1) and probed with a histidine-rich protein cDNA probe detected a 7.7-kilobase (kb) and 1.8-kb fragment, respectively. The 7.7-kb *Hind*III fragment was cloned

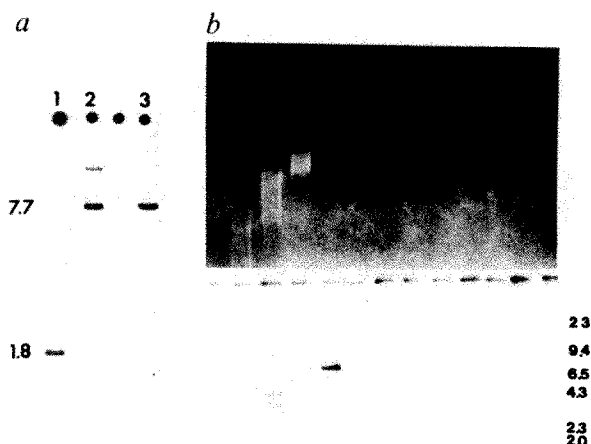


Fig. 1 *a*, Genomic cloning of the gene for histidine-rich protein into λ L47.1. *b*, Preparative agarose gel electrophoresis of *P. lophurae* DNA to enrich for the 7.7-kb *Hind*III fragment containing the gene.

Methods: *a*, *P. lophurae* was maintained in infected ducklings²⁶. DNA was isolated from infected duck erythrocytes by the saponin lysis procedure^{27,28}. 2 μ g of high-molecular weight DNA were digested with *Eco*RI (*a*, lane 1) or *Hind*III (*a*, lane 3) and the resulting fragments were separated on a 0.75% agarose gel and transferred to nitrocellulose paper²⁹. The gel was probed with nick-translated histidine-rich protein cDNA labelled to a specific activity of 2×10^8 c.p.m. μ g⁻¹ and hybridized in 50% formamide, 10% dextran sulphate, $5 \times$ SSC, $1 \times$ Denhardt's, 200 μ g ml⁻¹ of salmon sperm DNA at 40 °C for 16 h. Nonspecific hybridization was washed off in $0.1 \times$ SSC, 0.1% SDS at 54 °C and the filters exposed to Kodak XAR film with Dupont Lightening Plus intensifying screens at -70 °C for 4-16 h. The specific 1.8-kb *Eco*RI and 7.7-kb *Hind*III fragments which hybridize to the cDNA probe are indicated. *a*, Lane 2: DNA isolated from clone 8A, digested with *Hind*III and co-electrophoresed with *P. lophurae* DNA to demonstrate that an intact fragment had been cloned. *b*, 100 μ g of *P. lophurae* DNA were digested to completion with *Hind*III and fractionated on a Bulls Eye Electrophoresis apparatus (Hofer Scientific). Fractions were collected, and aliquots analysed on a 0.75% agarose gel (*b*, upper panel). The DNA fragments were transferred to nitrocellulose paper and probed with the cDNA probe. DNA fragment sizes are indicated in kb. A peak fraction containing the 7.7-kb *Hind*III fragment is visible in the lower panel. This fraction was ligated into λ L47.1 *Hind*III arms, packaged *in vitro*³⁰ and used to infect LE 392. 1×10^5 recombinant phage were obtained from a microgram of *P. lophurae* DNA. 5×10^4 phage were screened by *in situ* hybridization³¹ with the nick-translated cDNA probe; a positive obtained, referred to as 8A, was plaque-purified and DNA isolated from this phage was mapped against *P. lophurae* DNA (*a*, lane 2).

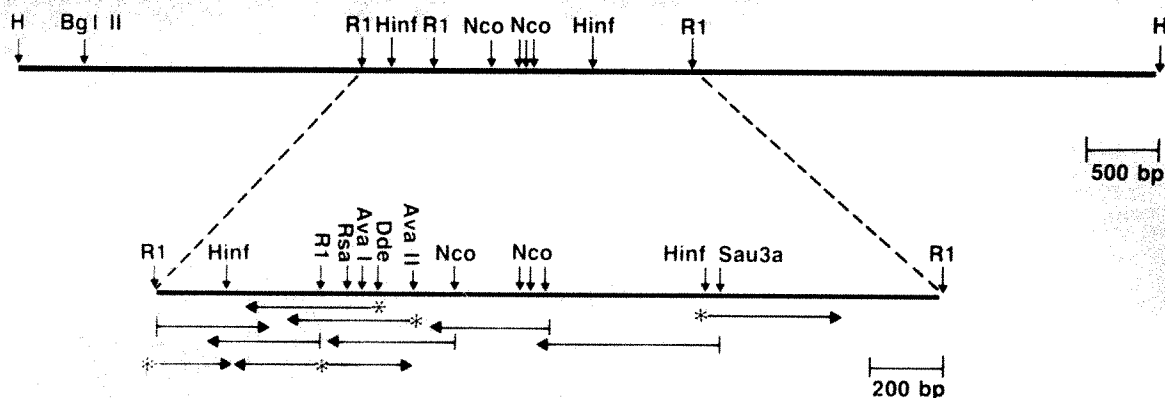


Fig. 2 Restriction map analysis and sequencing strategy for clone 8A of the histidine-rich protein gene. The 7.7-kb *Hind*III fragment, cloned as described in Fig. 1 legend, was mapped both within the phage and from a pBR322 subclone. Fragment sizes of the cloned DNA were compared with *P. lophurae* DNA. DNA sequencing analysis was performed using both the dideoxy method of Sanger and Coulson³², indicated by arrows ending with vertical lines, and by the chemical method of Maxam and Gilbert³³, indicated by arrows ending with stars. Fragments were obtained both from the phage clone 8A and from the pBR322 subclone. Repeat sequences obtained with the same fragment or M13 clone are not shown. The sequence obtained from the 3' *Hinf* site to the 5' *Nco* site was derived from two M13 clones which could be obtained in only one orientation due to the instability of the sequences in *Escherichia coli*. Multiple independent isolates of these clones were sequenced to generate the data shown in Fig. 3. This overlap, however, may be subject to some ambiguity.

ments by enriching *Hind*III-digested *P. lophurae* DNA by preparative agarose electrophoresis (Fig. 1b). Screening of 50,000 recombinant phages with the cDNA probe yielded a positive clone identified as 8A. Propagation of 8A in the bacterial vector LE 392 resulted in spontaneous deletion of the cloned insert, leading to segregation of the phage into two populations separable on CsCl density gradients. Only higher density, full-length phage particles were used for subsequent studies. *Hind*III digestion of clone 8A (Fig. 1a, lane 2) yielded a 7.7-kb fragment which co-migrated with the genomic *Hind*III fragment (Fig. 1a, lane 3). No deletion was apparent in this higher density phage fraction. The 7.7-kb *Hind*III fragment containing the genomic sequence of the protein was subcloned into pBR322 and propagated in HB101. Spontaneous deletion of the insert occurred in this system as well. A subclone, 8A-1, showed a 3.0-kb deletion in the 3' *Eco*RI-*Hind*III fragment of clone 8A (Fig. 2). *Eco*RI digests of clone 8A, 8A-1 and *P. lophurae* DNA revealed a co-migrating 1.8-kb fragment when probed with the cDNA probe (data not shown). *Bgl*II-*Nco*I digests of these DNAs revealed co-migrating 2.85-kb and 200-base pair (bp) fragments when probed with the 1.8-kb *Eco*RI fragment. Similarly, *Hinf*I digests of these DNAs revealed a common 1.35-kb fragment when probed with the 1.8-kb *Eco*RI fragment. These data confirm that no deletion or rearrangement had occurred in the 7.7-kb *Hind*III fragment in clone 8A or in the sequences extending from the *Bgl*II site 5' of the gene to the *Eco*RI site 3' of the gene in the subclone 8A-1.

Gene structure

The DNA sequencing strategy for the region encoding the histidine-rich protein (Fig. 2) revealed a sequence of 1,648 nucleotides (Fig. 3). An open reading frame coding for 328 amino acids (225 are histidine) extends from nucleotide 491 to nucleotide 1,487. Starting at nucleotide 563, we find a sequence identical to the recently reported sequence of 25 NH₂-terminal residues of mature histidine-rich protein¹³. There was no methionine in our reading frame, however, suggesting that the gene is interrupted and that the amino-terminal portion of the protein is located on another exon. Further analysis revealed another open reading frame with a putative initiation codon located at nucleotides 291-293, ending in a stop codon (nucleotide 366-368), and immediately preceded by a splice sequence AAGCGTAAG (boxed in Fig. 3) similar to the consensus 5' splice sequence, AAGGTAAG¹⁴. Similarly, a 3' splice sequence, TTATAG (boxed in Fig. 3), similar to the consensus 3' splice sequence, TTXCAG¹⁴, is found immediately adjacent to the next exon. We designed experiments to identify the predicted intron.

To demonstrate that the genomic DNA is not contiguous with its mRNA, we performed S₁ nuclease mapping. A 427-bp *Ava*I-*Hinf*I fragment (Fig. 4) spanning the intron-exon border was 5' end-labelled on the noncoding strand. After strand separation, the 5'-labelled strand was annealed to *P. lophurae* RNA. S₁ nuclease treatment of this hybrid yielded a 130-bp protected fragment (Fig. 5a, lane 2), consistent with a discontinuity between the RNA and the genomic DNA at nucleotide 491, at the intron-exon border.

To identify the break point between the DNA and RNA sequences specifically, we carried out primer extension studies. A 42-bp *Ava*I-*Rsa*I fragment (Fig. 4) was labelled on the 5' end of the noncoding strand. After strand separation, the 5'-labelled strand was annealed to *P. lophurae* RNA. A cDNA was synthesized in the presence of dXTPs and reverse transcriptase and the resulting 600-bp fragment was isolated and sequenced by chemical degradation. Parallel sequences were obtained from a 427-bp *Ava*I-*Hinf*I fragment (Fig. 4) labelled at the *Ava*I 5' end. The primer-extended sequence (Fig. 5b, lane 2) diverges from the genomic sequence (Fig. 5b, lane 1) at the position of the putative intron (arrow, Fig. 5b). The sequence derived by primer extension (Fig. 5c) agrees precisely with the 5' exon sequence (Fig. 3). A 5'-untranslated sequence can be read from the primer extension experiment for at least 150 nucleotides 5' of the exon, which similarly is in agreement with the genomic sequence. Additional S₁ mapping studies (data not shown) demonstrate that the 5'-untranslated sequence extends for at least 300 nucleotides beyond the open reading frame for the signal peptide. The precise 5' end of the untranslated mRNA sequence has not been identified. R-loop mapping of the genomic clone and mRNA (data not shown) demonstrates a 1,400 nucleotide R-loop, corresponding to the histidine-rich exon and 3'-untranslated sequences. Therefore, the length of the 3'-untranslated sequence is deduced to be 400 nucleotides (Fig. 4). The size of the mRNA has been identified by Northern gel analysis to be 2,200 nucleotides¹², suggesting that the 5'-untranslated sequence is approximately 700 nucleotides long.

Amino acid sequence

The prepro-protein contains 351 amino acid residues and, in its unglycosylated form, has a calculated *M_r* of 49,000. The amino acid sequence is numbered beginning at -47 for the signal peptide, at -24 for the pro-peptide and at +1 for the mature protein.

The assignment of the methionine at -47 as the initiating methionine of the prepro-protein needs to be confirmed by amino acid sequencing of the primary translation product. Our principal argument in support of this assignment is that the 24

GAATCTATTATTTAACTTATTTAAATATATTTCTTTTAAAGAAATATTTATTCATTTTATGTTTT	71
TTTAAACATTTTATTTTATTTTATTTTGTGATAAATAAAATATCCATTCTAAGGCACTGCTGTTTATTTT	142
AAGATAACAATATATTTATATCTTTAAATATACCATAATTAACATGGGATTCATCAATGCCAACAATA	213
ATATTAGGATTTCCATGAGGGAGGTCATCGGCCACACGCCCTTAAATCAAATCAAAAAAAGGGAAA	284
Met Phe Thr Ser Leu Lys Lys Val Ala Thr Phe Ser Phe Leu Val Trp	
GGAAGT ATG TTT ACT TCT CTT AAG AAA GTT GCT ACT TTT TCC TTT CTA GTT TGG	338
Ile Ser Gln Tyr Ser Gly Ser	
ATA TCT CAA TAT TCA GGA AGC GTAAGATAAAGCTAATGATAAAATATATATATGTTATGTTT	402
TTCTTATTTACACTCTTAAATTAATAATGATAATATAAAAAAAGAAAAATCTTTAATTTAATT	473
Asn Ser Cys Ser Ser Ser Leu Val Lys His Ile Pro Gln	
TTTATTTATTTATATG AAT TCA TGT AGC TCC TCA CTT GTC AAA CAT ATC CCA CAA	529
Thr Gly Ser Asn Leu Thr Phe Asp Arg Val Leu Val Glu Asp Thr Val His Pro	
ACG GGG TCA AAC TTA ACA TTT GAT AGA GTT TTA GTA GAA GAT ACA GTA CAC CCT	583
Glu His Leu His Glu Glu His His His His His Pro Glu Glu His His Glu Pro	
GAA CAT CTC CAT GAA GAA CAC CAC CAT CAT CCC GAG GAA CAT CAT GAA CCA	637
His His Glu Glu His His His His His Pro Glu Glu His His Glu Pro His His	
His His GAA CAT CAC CAT CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC	691
Glu Glu His His His His His Pro His Pro His His His His His His His Pro	
GAA GAA CAT CAC CAT CAC CAT CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC	745
Pro His His His His His His His His His His His His His His His His	
CCT CAT CAT CAT CAC CAC CTA GGA CAT CAT CAT CAT CAT CAT CAT CAT CAT	799
His His His His His Glu Glu His His His His His His His His His His His	
CAC CAT CAT CAC GAA GAG CAT CAT CAC CAC CAC CAC CAC CAC CAC CAC CAC	853
His Glu Glu His His His His His His His His His His His His His His His	
CAC GAA GAG CAT CAT CAC CAC CAT CAC CAC CAC CAC CAC CAC CAC CAC CAC	907
His His His Leu Gly Tyr His His His His His His His His His His His His	
CAC CAT CAT TTA GGA TAT CAC CAT CAC CAT GCT CCA CAC CAT CAT CAC CAC	961
His His Ala Pro His His His His His His His His His His His His His His	
CAT CAT GCT CCA CAC CAT CAT CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC	1015
His His His His His Ala Pro His His His His His His His His His His His	
CAC CAC CAC CAT GCT CCA CAC CAT CAT CAC CAC CAC CAC CAC CAC CAC CAC	1069
His His His His His His Gly His His His His His His His His His His His	
CAT CAC CAC CAC CAT GGT CAC CAC CAT CAT CAC CAC CAC CAC CAC CAC CAC	1123
His His His His His His His His His His His His His His His His His His	
CAC CAT CAT CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC	1177
Asp Ala His His His His His His His His His His His His His His His His	
GAT GCA CAT CAC CAC CAC CAT CAT CAT CAC GAT GCA CAC CAC CAC CAC CAC	1231
His His Asp Ala His His His His His His His His His His His His His His	
CAT CAT GAT GCA CAT CAC CAC CAC CAC CAT CAT CAC GAT GCA CAT CAC CAC	1285
His His His His Asp Ala His His His His His His His His His His His His	
CAT CAT CAT CAC GAT GCA CAC CAC CAC CAC CAT CAT CAT CAC GAT GCA CAC	1339
His His His His His Asp Ala His His His His His His His His His His His	
CAC CAT CAT CAC GAT GCA CAT CAC CAC CAC CAC CAT CAT CAT CAC GAT GCA	1393
His His His His His His His His His His His His His His His His His His	
CAC CAC CAC CAT CAT CAC GAT GCA CAC CAC CAC CAC CAT CAT CAT CAC GAT	1447
Ala His His His His His His His His His His His His His His His His His	
GCA CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC	1509
TTATGTGAATTTTAAATAGAATAAGCATTATATATATATATATATATATATATATATATATAT	1580
TACGTAGACATTTTCTAGTCTTTTATCTTTTTCAGTGATTCATTTCTTCAATTCATGATC	1648

Fig. 3 Nucleotide sequence of the gene for histidine-rich protein and the predicted amino acid sequence of the prepro-protein. 1,648 nucleotides are shown, corresponding to the region indicated in Fig. 2. The predicted amino acid sequence is numbered beginning at -47 for the signal peptide and at -24 for the pro-peptide. The mature protein begins at amino acid 1 and corresponds to the N-terminal amino acid sequence obtained by Howard *et al.*¹³. ▽, A potential signal peptidase cleavage site^{15,16}; ▼, the processing site between the pro-protein and the mature protein. A potential site for Asn-linked glycosylation in the pro-peptide portion is overlined. The 5' and 3' putative splice sites are boxed and demonstrate considerable homology to the consensus splice sequences.

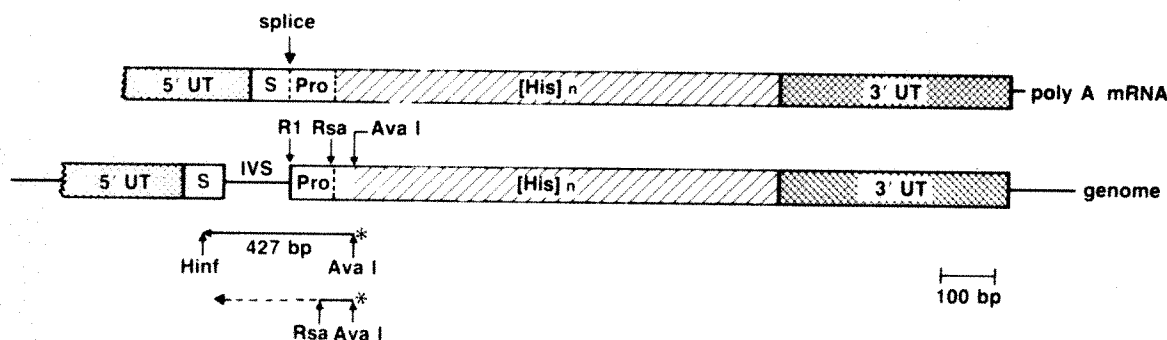


Fig. 4 The organization of the histidine-rich protein DNA, mRNA and the expressed protein. A schematic representation of the gene and its transcript are shown with the intervening sequence (IVS) indicated in the gene. The protein is divided into a pre (signal) sequence, a pro-peptide and mature protein. Transcribed and untranslated (UT) sequences are indicated. The precise 5' initiation site of the mRNA has not been determined and is indicated by the sawtooth line at the 5' end of the DNA and mRNA. A 427-bp *Ava*I-*Hinf*I fragment derived from the genomic clone 8A is indicated, as is a 42-bp *Ava*I-*Rsa*I fragment used in the primer extension studies described in the text. The fragments were derived from the non-coding strand and were 5' end-labelled *.

residue-long sequence following the methionine at -47 is highly characteristic of a signal peptide containing a stretch of hydrophobic residues and two charged residues (Lys -42 and Lys -41) preceding this hydrophobic stretch. The only other in-frame initiation codon further upstream (nucleotides 199-201) would encode a sequence that is not characteristic of a signal peptide.

The assignment of the signal peptidase cleavage site between residues -25 and -24 is based on consensus features which have been proposed for this site^{15,16}. Definitive assignment of this site must await NH₂-terminal sequencing of *in vitro* synthesized histidine-rich protein that is segregated by dog pancreas rough microsomes⁹ and that can be expected to have its signal peptide removed by the dog pancreas microsomal signal peptidase at the correct site¹⁷.

The signal peptide is followed by a pro-peptide that shows a consensus glycosylation site at Asn -8. Histidine-rich protein synthesized in a cell-free system and segregated by dog pancreas microsomes was found to be core-glycosylated⁹. The fact that the mature protein is not core-glycosylated suggests that the core-glycosylated pro-peptide portion is removed on transport from the rough endoplasmic reticulum to the membrane-bound granules. There are precedents for the existence of core-glycosylated propeptides in the synthesis of other secretory proteins, the mature forms of which are not core-glycosylated¹⁸. Whether the propeptide of the histidine-rich protein is also glycosylated *in vivo* remains to be shown.

The most striking features of the sequence organization of the mature protein are the tandemly repeated elements. The histidine-rich sequence begins at amino acid 12 with a sequence (Glu)₂-(His)₅-Pro-(Glu)₂-(His)₂-Glu-Pro-(His)₂ repeated once. Amino acids 44-76 appear to have a degenerate repeat of the form X-X-(His)₅-X-X-(His)₇ repeated once; this is followed by two repeats of the sequence (Ala)₂-(His)₅-(Glu)₂-(His)₆-(Ala)₂, five repeats of the sequence Ala-Pro-(His)₈, three repeats of the sequence Gly-(His)₉, and finally nine repeats of the sequence Asp-Ala-(His)₈, with one copy of the sequence Asp-Ala-(His)₇.

On the assumption that there is no trimming at the COOH terminus, the mature histidine-rich protein is calculated to have a relative molecular mass of 43,000, containing 74% histidine.

The predominant residues in the mature protein other than histidine are Ala, Glu, Pro and Asp; Asn, Arg, Cys, Gln, Ile, Lys, Met and Ser are completely absent. These data are in close agreement with the previously reported amino acid composition of mature histidine-rich protein. This exceptional amino acid composition is probably responsible for the abnormal migration of the protein on SDS-polyacrylamide gel electrophoresis⁹.

Site of the 5' exon

Two synthetic oligodeoxynucleotide probes comprising the 5' exon were synthesized. These oligonucleotides were 5' end-labelled and used as probes to determine the genomic organiz-

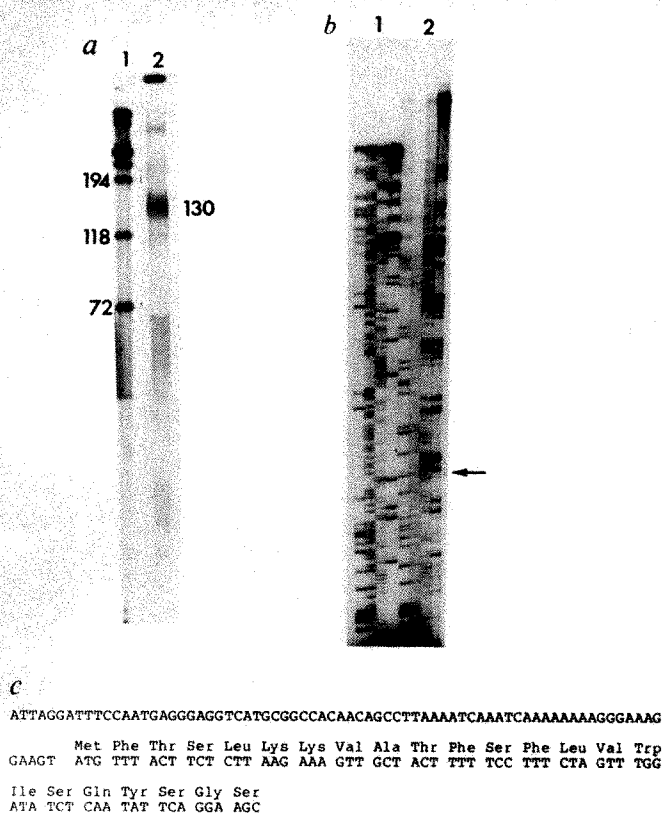
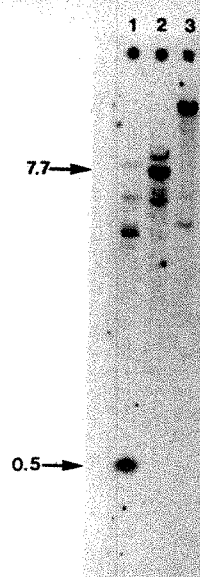


Fig. 5 Mapping the intron in the gene for histidine-rich protein. *a*, S_1 nuclease mapping; *b*, primer extension. Lane 1, an autoradiogram of the DNA sequencing gel for the genomic *AvaI-Hinf* fragment; lane 2, the primer-extended fragment. *c*, cDNA sequence of the 5' exon and untranslated region deduced from the primer-extension experiment in *b*. The predicted amino acid sequence is shown above the nucleotide sequence, identical to the genomic sequence in Fig. 3. The sequence beyond the break point is shown, corresponding to the 5' exon sequence. (Identical 3' sequences for the two fragments are not shown.)

Methods: *a*, A 427-bp *AvaI-Hinf* fragment, spanning the intron-exon border, was isolated (see Fig. 4), labelled on the 5' *AvaI* end and strand-separated on a 10% polyacrylamide gel³⁴. 2×10^4 c.p.m. were co-precipitated with 10 μ g of *P. lophurae* mRNA in 70% ethanol at -20°C for 16 h. The precipitate was resuspended in 30 μ l of 80% formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4 and 1 mM EDTA. The reaction was incubated at 80°C for 15 min, rapidly cooled to 50°C , then incubated at 50°C for 3 h. The reaction was diluted with 0.3 ml of S_1 buffer (0.28 M NaCl, 0.05 M NaAc, pH 4.5, 4.5 mM ZnSO_4) and 300 units of nuclease S_1 were added. The reaction was incubated for 30 min at 37°C and stopped by adding 10 μ l 0.5 M EDTA. After phenol-chloroform extraction, the S_1 nuclease-resistant material was ethanol-precipitated, resuspended in 95% formamide and dyes and fractionated on a 10% acrylamide, 7 M urea sequencing gel. The gel was dried and autoradiographed for 16 h. Size markers (lane 1) are indicated, as is the position of the protected DNA fragment at 130 nucleotides (lane 2). *b*, A 42-bp *AvaI-Rsa* fragment was labelled on the *AvaI* end, strand-separated and coprecipitated with 10 μ g of *P. lophurae* mRNA in 70% ethanol at -20°C for 16 h. The pellet was resuspended in 50 μ l of 70% formamide, 1 mM EDTA, 0.5 M NaCl, 40 mM PIPES, pH 6.4 and incubated for 16 h at 56°C . The hybridized material was collected by ethanol precipitation and resuspended in 50 μ l of 0.1 M NaCl, 1 mM EDTA, 10 mM Tris, pH 8.3. An extension reaction of 100 μ l volume containing 50 mM Tris pH 8.3, 10 mM MgCl_2 , 1 mM of each of the four dNTPs, 10 mM dithiothreitol and 100 units of reverse transcriptase was incubated at 42°C for 1 h and terminated by the addition of EDTA to 10 mM, followed by phenol extraction and ethanol precipitation. The pellet was resuspended in formamide-dye loading buffer and fractionated on a 10% DNA sequencing gel. The resulting 600-nucleotide fragment was eluted from the gel and subjected to DNA sequencing by the method of Maxam and Gilbert³³. The arrow indicates the break point of the two sequences, which corresponds to the intron-exon junction (see Fig. 3).

Fig. 6 Multiple occurrence of the signal exon sequence in the *P. lophurae* genome. Two synthetic oligodeoxynucleotides with the sequences 5' CCAAAGTAGAAAGGAAAAAGTAGC 3' (complementary to nucleotides 315-338) and 5' AACTTTCTTAAGAGAAGTAAACAT 3' (complementary to nucleotides 291-314) which correspond to the signal exon sequence (see Fig. 3) were synthesized, 5' end-labelled to a specific activity of 1×10^8 c.p.m. μg^{-1} and purified on a 15% acrylamide, 7 M urea gel. DNA from *P. lophurae* was digested with *EcoRI* (lane 1), *HindIII* (lane 2) and *BglII* (lane 3), transferred to nitrocellulose and hybridized with 10^7 c.p.m. of the oligonucleotide probes at 50°C in $6 \times \text{SSC}$, 0.1% SDS, $10 \times \text{Denhardt's}$ and 200 mg ml^{-1} salmon sperm DNA for 16 h. The filter was washed twice at 25°C in $6 \times \text{SSC}$, 0.1% SDS and twice at 37°C in $6 \times \text{SSC}$, 0.1% SDS. The filter was dried and exposed to Kodak XAR film at -70°C with an intensifying screen for 16 h. The 0.5-kb *EcoRI* and 7.7-kb *HindIII* gene fragments are indicated.



ation of the 5' exon in the *P. lophurae* genome (Fig. 6). In addition to the expected 0.5-kb *EcoRI* fragment and the 7.7-kb *HindIII* fragment (see Figs 1 and 2), multiple DNA fragments were detected with the enzymes chosen to digest the *P. lophurae* DNA, whereas only a single DNA fragment hybridized to the cDNA (see Fig. 1), indicating that the 5' exon sequence is present in multiple copies in the genome. Hybridization at reduced stringency using the 1.8-kb *EcoRI* fragment failed to detect additional cross-hybridizing sequences (data not shown).

To determine whether any of these cross-hybridizing sequences are closely linked to the gene for histidine-rich protein, clone 8A and subclone 8A-1 were digested with multiple restriction endonucleases; no additional DNA fragments hybridized with the oligonucleotide probes (data not shown), establishing that >2.0 kb must separate the signal peptide exon from the cross-hybridizing sequences.

Conclusions

Tandem repeats have been reported for other *Plasmodium* proteins in which the amino acid sequences derived from their cDNA and genomic clones reveal only one type of repeat. It remains to be seen whether these proteins, like histidine-rich protein, show several distinct repeats. The sequences of the repeats differ in all these proteins.

The surface antigens of *Plasmodium falciparum* may be a family of molecules comprised of repeating, antigenically distinct subunits¹¹. If histidine-rich protein becomes a surface molecule of *P. lophurae*, it must be secreted. Indeed, its early biosynthetic forms resemble those of secretory proteins^{19,20}, but it has not yet been established whether, and at what point of parasite development, secretion of the protein occurs. If secretion does occur, it would be followed by disassembly of the protein aggregates (perhaps by partial proteolysis) before association with the parasite surface.

The 131-nucleotide intron separating the signal peptide-encoding exon from the exon coding for histidine-rich protein demonstrates highly conserved splice sequences. Of interest is the occurrence of a sequence element at nucleotides 458-466 (TCTTTAACA), 22 nucleotides upstream of the 3' splice site, which is very similar to the splicing signal sequence in yeast (TACTAACA)^{21,22}. This TACTAACA box has been found 20-55 nucleotides upstream of the 3' splice site in all split protein-coding nuclear genes of *Saccharomyces cerevisiae* and is an essential element of the yeast splicing mechanism. These data suggest that *Plasmodium* possesses the cellular machinery to process precursor RNAs into mature message, perhaps using a mechanism similar to that of yeast.

We have shown here that the sequence comprising the signal peptide-encoding exon is present in multiple copies in the *P. lophurae* genome. Thus far, the conservation of a signal sequence

at the DNA level is unique to *P. lophurae* and without precedent in other genomes. This finding is particularly interesting when viewed in the context of a 35-bp long sequence that occurs in a tandem array in the genome of trypanosomes. This sequence can be translocated and is found associated with mRNAs coding for variable surface glycoproteins²³⁻²⁵ and other cellular mRNAs³⁵. This 35-bp sequence of unknown function occurs at the 5'-untranslated end of these mRNAs. Our data, presented here, demonstrate the redundancy of a 5' exon for another protozoan and suggest that a coding sequence of known function may be used by a diverse population of genes. Investigations are under way to identify these signal peptide-encoding exons

Received 11 June; accepted 11 September 1984.

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in the plasmodial genome and to assign the exons to expressed mRNAs.

This work was supported by grants to J.V.R. from The Kleberg Foundation, The Charles E. Culpeper Foundation and The Stanton Fund and to G.B. from the NIH (PHS GM 27155) and the Rockefeller Foundation. We thank Peter Model for synthesizing the oligonucleotides used in this study and for helpful conversations. We appreciate the comments and suggestions of Steven Meshnick and George Cross, the unpublished results of Russell Howard and Michael Wallach, the technical assistance of Elizabeth Schultz and Wendy Whyte, and thank Karen Yates, Mary Wentzler and Gisele Nimic for preparing the manuscript.

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LETTERS TO NATURE

A runaway instability in thick accretion disks?

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Others have proposed¹ that thick disks of material accreting around black holes may be subject to a runaway instability. If the mass of the disk exceeds a critical value ($m_{\text{disk}} > 0.01 M_{\text{hole}}$), the disk drains into the hole on a dynamical time scale. We report here a study of this effect, using models of non-self-gravitating disks in the Kerr metric, which enables proper allowance to be made for the effects of black-hole rotation and the increase in the spin of the hole as it accretes. We conclude that there is no runaway instability in these systems.

The construction of disk models in the standard approach, due to the school of Abramowicz, is straightforward^{2,3}. For an axisymmetric stationary space-time, the metric has the form⁴

$$ds^2 = -e^{2\nu} dt^2 + r^2 B^2 \sin^2 \theta e^{-2\nu} (d\phi - \omega dt)^2 + e^{2\mu} (dr^2 + r^2 d\theta^2) \quad (1)$$

where ν , μ , ω and B are functions of polar coordinates r and θ . If the disk has negligible influence, equation (1) will be the Kerr metric. The specific angular momentum of the disk material, l , is given an assumed distribution; but here l is taken to be constant, for reasons discussed below. The disk material is assumed to be barytropic; here it has the equation of state of an isentropic radiation-dominated plasma, with $p \propto \rho^{4/3}$ (ρ is the rest-mass density, and the total mass energy density is $\epsilon = \rho + 3p$). However, these assumptions are not critical. By assuming only azimuthal motion and that l is constant, the hydrodynamic equations can be integrated to give²

$$\int_0^p \frac{dp}{\epsilon + p} = W - W_0 = -\ln u_i / u_0 \quad (2)$$

where W is the combined gravitational and centrifugal potential and u_i is the appropriate component of the four-velocity, which

has a physical meaning as the specific 'energy at infinity' and is calculated from l and the metric². The quantities u_0 and W_0 are those evaluated at the inner edge of the disk. For the equation of state assumed here, equation (2) becomes

$$\rho = K \left(\frac{u_0}{u_i} - 1 \right)^3 \quad (3)$$

defining K as a parameter that depends (inversely) on the entropy. If K is large, the disk material is cool. The specific angular momentum, l , must lie between l_{ms} , the angular momentum of the innermost stable orbit, and l_{mb} , the angular momentum of the marginally-bound circular orbit. If $l \sim l_{\text{ms}}$, the disk is very small, whereas if $l \sim l_{\text{mb}}$ the disk is very thick.

The potential W has a cusp, due to relativistic effects, along a ring in the equatorial plane (see, for comparison, Fig. 1a of ref. 1). This cusp defines a critical equipotential surface (analogous to the Roche lobe in binary systems) within which a stable disk must be confined, otherwise material will flow into the hole. Using the Kerr metric and the integral for the mass of a rotating fluid configuration (equation (3.49) of Bardeen⁴), the mass of a non-self-gravitating disk that just fills its critical surface can be written as

$$m_{\text{disk}} = K m_c(M, J, l) \quad (4)$$

where m_c is a function of the hole mass M , its angular momentum J , and the specific angular momentum of the disk material l . Equation (4) can be solved by straightforward numerical integration.

Consider now what happens if a mass δm is transferred from the disk to the hole. The mass of the hole rises by δm , and, as the material has specific angular momentum l , the angular momentum of the hole rises by $l\delta m$. The metric around the hole changes and so does the four-velocity component u_i in the flow. If the accretion process is axisymmetric, we expect the specific angular momentum to remain constant. There are three flow variables in general relativity that reduce to the newtonian specific angular momentum in that limit: these are u_ϕ (four-velocity component), $l = -u_\phi / u_i$ and $j = (\epsilon + p)u_\phi / \rho$. The equations of motion imply that if l is constant, then j is constant. In an axisymmetric flow, j is conserved along the flow⁵. Thus, if j is constant at the start, any axisymmetric accretion will leave

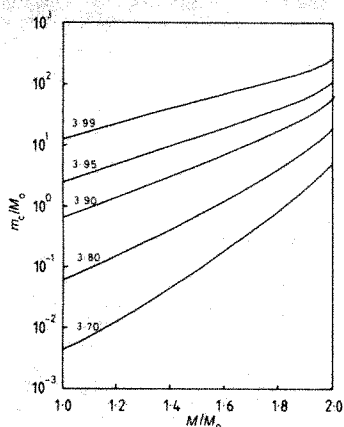


Fig. 1 Curves of the critical surface mass function, $m_c(M, J, l)$ as a function of increasing hole mass, M : m_c increases along the accretion trajectories defined by various values (given) of j/M_0 . ($J=0$ when $M=M_0$ —the initial mass.) As $dm_c/dM > 0$ everywhere, these curves show that all non-self-gravitating disks are stable with respect to the runaway instability.

j with the same value, independent of the mass-transfer process. This simplification is the reason for choosing l to be constant. During accretion, u_i decreases, implying that l increases, so the result of accretion is a disk with the same (constant) j and a larger l .

The runaway instability arises when a disk that just fits its critical surface loses a little of its matter to the hole. If the critical surface shrinks, the remaining matter may not fit in the surface, whereby more mass is lost to the hole, and so the process continues.

In the accretion process, unless energy is lost to infinity, the total gravitational mass of the system ($M_t = M + m_{\text{disk}}$) is constant. Consider a disk just filling its critical surface, for which $m_{\text{disk}} = m_c(M, J, l)$, that loses δm to the hole. The total mass of a system comprising a hole of increased mass and a critical surface-filling disk, with the same entropy (assumed constant in the process) and the same j , is $M'_t = M + \delta m + KM_c(M + \delta m, J + \delta J, l + \delta l)$. If $M_t > M'_t$, then the disk does not fit the new critical surface and an instability arises. This condition can be shown to be identical to that at which the rest mass fits the critical surface. A set of M, j, l such that $dJ/dM = l$, and j is constant, defines the 'accretion trajectory' that a black hole and disk will follow, if mass is gained from the critical surface. Then the condition for stability along an accretion trajectory can be stated as

$$K \frac{dm_c}{dM} > -1 \quad (5)$$

The ranges of parameters that must be considered are $0 \leq J/M^2 \leq 1$ and $l_{\text{ms}} \leq l \leq l_{\text{mb}}$. It is found, throughout this parameter space, that $dm_c/dM > 0$, so all non-self-gravitating disks with constant l are stable with respect to the runaway instability. Figure 1 illustrates this in showing m_c always increasing along the accretion trajectories.

Why does our result differ from that of Abramowicz *et al.*¹? In simple terms, the size of the critical surface is governed by the ratio $(l - l_{\text{ms}})/(l_{\text{mb}} - l_{\text{ms}})$: when it is large, the disk is large. As the hole gains mass, l_{ms} tends to increase; if l is fixed, as it is in the pseudo-newtonian calculation, the ratio decreases, and the critical surface shrinks. The inclusion of relativistic effects changes this in two ways: first, as the hole accretes matter, it spins up, and a spinning hole has a lower l_{ms} than a non-spinning hole; second, l increases as the accretion proceeds. These two effects combine to make the ratio rise rather than fall when mass is transferred. Self-gravity is unlikely to affect this result.

A recent paper⁶ shows that constant angular momentum (newtonian) disks are subject to a non-axisymmetric instability. However, it is unclear whether such a disk is completely dis-

rupted, and whether the instability applies to disks with a significantly non-uniform distribution in l . Stable axisymmetric disks may exist and our result shows that these will not be subject to the runaway instability. Specific angular momentum, l , must increase outwards, so when material is lost from the inner edge of the disk to the hole, it is replaced by material with a higher value of l , which is more stable. Thus a disk with non-uniform l is less subject to runaway instability than one with uniform l , which, in turn, is stable with repeat to this process.

I thank M. Abramowicz for useful discussions and the SERC for financial support.

Received 14 September; accepted 11 October 1984.

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Possible detection of a ring system in Saturn's magnetosphere

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The region of the magnetosphere of Saturn around 14 radii and 19 radii has been of special interest since the detection of anomalous dips in the low-energy plasma ion-density in these regions by the Voyager 1, Voyager 2 and Pioneer 11 spacecrafts. Lazarus *et al.* suggested that these density dips may be due to long-lived particulate or gaseous structures in the equatorial plane of Saturn¹. Baron and Elliot, using the charge-coupled device (CCD) imaging technique, found no evidence of any material existing between 10 and 36 Saturn radii². Their negative results, however, have set an upper limit on the extent of a possible material cloud with several assumptions regarding its nature and density. We have observed the occultation of the star SAO158913 from two stations in India following predictions by Mink³. This star showed symmetric dips in the light curves on 24 March 1984 (immersion event) and on 25 March 1984 (emersion event), suggesting the possible presence of particulate matter.

Figure 1 shows the path of the star relative to the planet. The outermost ellipse is the projection on the sky plane of a circular ring at 14 radii. The next ellipse inwards represents the projection of the observed ring at ~12.5 radii. The visible inner rings are also shown for comparison.

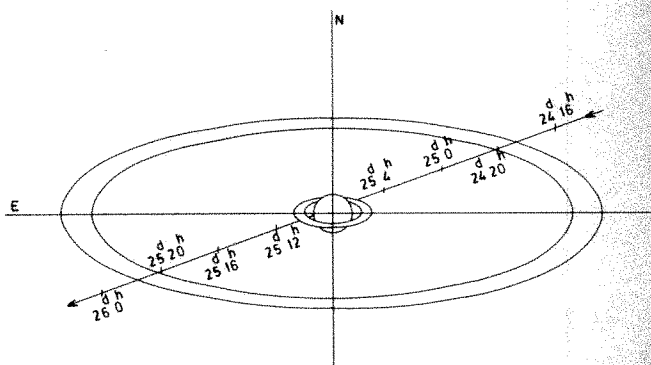


Fig. 1 Path of the star relative to the planet. The outermost ellipse is the projection on the sky plane of a circular ring at 14 radii. The next inner ellipse represents the projection of the observed ring at ~12.5 radii. The visible inner rings are also shown for comparison.

Observations were made on the 102 cm reflector at Kavalur⁴ (latitude +12° 34.58', longitude -5 h 15 min 19.6 s) using an EMI 9658 photomultiplier cooled with dry ice. Continuous d.c. recording (strip-chart recorder) and pulse counting using instrumentation developed locally were performed simultaneously on both nights. The continuous recording had a time constant of 1 s whereas a 5 s integration time was used in the photon counting mode. The observatory had photometric

observing conditions operating throughout 24 March and until 22 h 30 min UT on 25 March, as confirmed by steady photoelectric records taken over almost the entire duration. Observations on 24 March were made without a filter, whereas a standard B-filter [BG 12(4 mm) + BG 18(2 mm) + GG 4(1 mm)] was introduced on 25 March. A circular diaphragm of 12.4 arc s diameter was used on both nights.

The observations were made at night at Kavalur from 18 h 19 min to 21 h 25 min on 24 March, and from 20 h 3 min to 22 h 30 min on 25 March. On both nights the light curve was steady except for one brief interval each night lasting for ~42 min. Figure 2a and b shows the 5-s counts for these periods on the two nights after subtraction of the sky counts. The appearance of curve b has been reversed so that the structures of possible occulting material clouds can be easily compared on either side of the planet. Figure 3 shows the variation in star light as recorded by the strip-chart recorder. Gradual variation in intensity during immersion is observed between 19 h 55 min and 20 h 33.5 min; two sharp features appear between 20 h 14 min and 20 h 20 min. Similarly, during emersion, gradual variation in intensity is observed between 20 h 5 min and 20 h 45 min, and the sharp features appear between 20 h 18 min and 20 h 24 min. Besides these, several other extinction features of lower magnitude are apparent in these records. On the immersion side (24 March), the broad-band photometry indicates a maximum drop of ~0.77 magnitude, whereas on the emersion side (25 March), B-filter measurements show a maximum extinction of ~0.15 magnitude.

Additional observations on 24 March, 1984, at Uttar Pradesh State Observatory, Naini Tal (latitude +29° 21.65', longitude -5 h 17 min 49.71 s), were made with a 104 cm reflector using a standard V-filter and an EMI 6094 s photomultiplier (thermo-electrically cooled to -20 °C). The photomultiplier output was recorded on a strip-chart recorder. Figure 2c shows the strip-chart data digitized at intervals of 5 s. The maximum decrease in stellar flux is ~0.23 magnitudes. No observations could be made from Naini Tal on 25 March 1984 because of cloudy sky conditions.

Assuming that the observed extinction is caused by material orbiting the planet in the equatorial plane, we have calculated the planetocentric distances of the occulting material. Approximate positions of the two bodies have been extrapolated from data in ref. 3, which differ somewhat from those indicated by

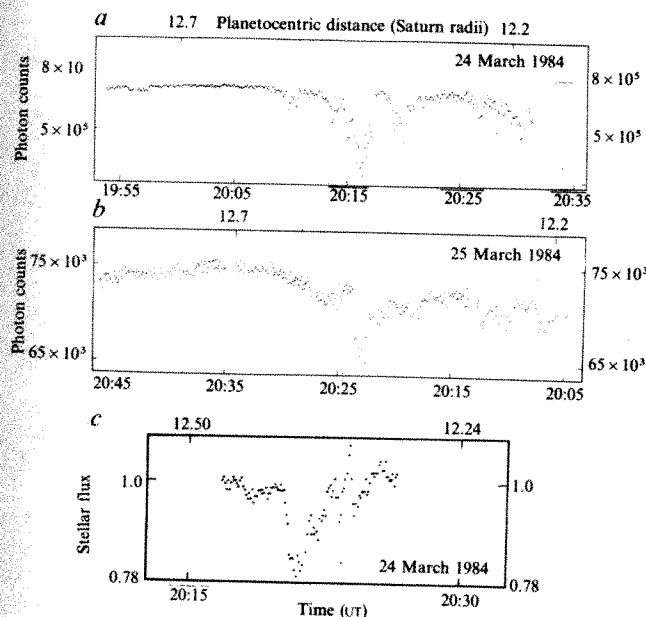


Fig. 2 Light curves: *a*, immersion on 24 March 1984 from Kavalur; *b*, emersion on 25 March 1984 from Kavalur, reversed for comparison with curve *a*; *c*, immersion on 24 March 1984 from Naini Tal. The Kavalur light curves were synthesized using the output of the photon counting unit with an integration time of 5 s. The normalized Naini Tal light curve was generated by digitizing the strip-chart record at 5-s intervals. The bottom and top scales along the abscissa give in each case the time in UT and the planetocentric distance in Saturn radii, respectively. Sky background has been subtracted in each case.

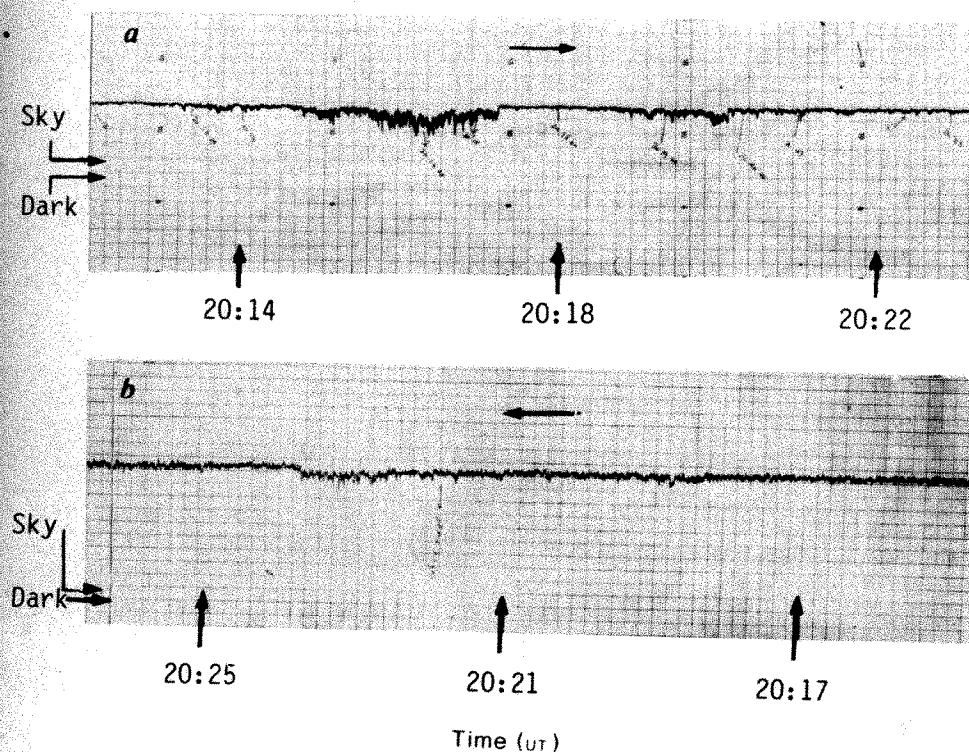


Fig. 3 Copy of the strip-chart record showing prominent features of the light curves obtained at Kavalur: *a*, immersion event on 24 March 1984 (20 h 12 min–20 h 23 min); *b*, emersion event on 25 March 1984 (20 h 26 min–20 h 14 min) reversed to allow direct comparison with the immersion light curve. The complete record is not shown (see text for description). Amplifier gains were different on the two days. Full scale corresponds to 10 mV.

the American Ephemeris record (1984) and the Smithsonian Astrophysical Observatory catalogue. More accurate values of the relative positions of the two bodies may become available when the times of A-ring occultations are determined. Hence our planetocentric distances are subject to modification by other events in the occultation not as yet taken into account. A typical error of 1.5" in declination and 0.1 s in right ascension, in either the star position or the planet's ephemerides, shifts the shadow centre of the planet by ~ 0.16 Saturn radii. Therefore, the calculated values for planetocentric distances may be in error by this amount.

The shapes of the main features of the three time profiles (Fig. 2) are similar, though the extinction magnitudes are different. The results indicate the probable existence of occulting material in orbit around Saturn at a planetocentric distance of ~ 12.5 Saturn radii. The planetocentric distances of the prominent features are not exactly the same in the three sets. This minor difference could arise from uncertainty in the relative positions of the star and the planet as mentioned above. The large discrepancy in the extinction magnitudes on the immersion side, as observed from the two sites, is puzzling, although the B-filter measurements on the emersion side almost agree with the V-filter observations on the immersion side. A realistic estimate of albedo from optical depth measurements depends on detailed modelling of the ring, which itself must await the results of further observations of occultation in other wavelength bands. An optical depth inferred from our observations would, in any case, generate an albedo of at least one or two orders of magnitude greater than the upper limit set by Baron and Elliot². This apparent inconsistency clearly emphasizes the necessity for more ground-based observations before further progress can be made.

Received 5 July; accepted 5 October 1984.

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A 154-day periodicity in the occurrence of hard solar flares?

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Since the launch of the Solar Maximum Mission (SMM) satellite on 1980 February 14, the Gamma-Ray Spectrometer (GRS)¹ has been monitoring the Sun in the energy range 0.3–100 MeV. Because of its large geometric area and good spectral resolution, the GRS has provided unprecedented spectral and temporal information about solar flares, of which 139 have been monitored. We report here on an analysis of the temporal distribution of these high-energy events to provide information on solar activity and find that, instead of being randomly distributed in time, these events have a tendency to occur in groups with a mean spacing of ~ 154 days (75 nHz) over the observing interval. A larger sample of flares (>500), with an X-ray classification of $\geq M 2.5$ recorded by the GOES satellites, showed a similar regularity.

The SMM satellite was launched into a near-circular orbit with an inclination of 28.5° and an altitude of 570 km. The orbit precesses, relative to the Earth–Sun line, with a period of ~ 48 days. Because of the decay of the orbit, the period of revolution has decreased. In November 1980, the fine-pointing attitude

stabilization malfunctioned, halting operations of the solar imaging instruments, but adequate stabilization was maintained for the GRS and two other instruments using magnetic torquing bars. This failure did not affect the GRS measurements because of the large field-of-view of the detector ($\sim 2\pi$ sr). South Atlantic anomaly passages and the occultation by the Earth's orbit limit the duty cycle for the observation of solar events to $\sim 50\%$. Since the launch, the instrument has functioned properly with no evidence of degradation. In the period October 1983–mid-April 1984, after the repair mission, data recovery was sporadic, though normal operations are now continuing. This paper deals only with observations made from 1980 February 19 to 1983 September.

The search for energetic photon solar flares was performed by visual screening of the counting rates at ~ 300 keV, the low-energy threshold of the GRS, and by a computerized analysis of fluctuations in the 350–800-keV energy band². The solar origin of any transient event identified in this search was confirmed by solar observations made at other wavelengths³. Over the period from the launch to September 1983, we have identified 139 solar flares emitting continuum radiation of energy ≥ 300 keV. Figure 1 shows the temporal distribution of these flares. The flare frequency was rather high throughout 1982 despite the fact that the mean sunspot number had decreased by $\sim 30\%$ from its maximum⁴. In fact, the interval of greatest energetic photon flare activity occurred in June/July and November/December 1982, 2–3 years after the sunspot maximum for this cycle. A time delay with respect to the sunspot maximum was also found in previous solar cycles for solar energetic particle events (ground level effects)⁵. The frequency of hard flare events decreased dramatically in 1983, reflecting the continuous decrease in the relative sunspot number and other activity monitors such as the 10.7-cm radio flux.

We also note from Fig. 1 that the flares tend to occur in groups which are spaced by about 5 months. Eight groups are visible with their centres near 1980 June and November, 1981 April and September, 1982 February, July and December and 1983 May. We have assessed the quantitative significance of these apparent groupings by calculating a Fourier power spectrum of the time distribution of flares in which each is treated as an independent event irrespective of its parent active region (Fig. 2a). Several peaks are seen, the most significant (labelled A, B and C in Fig. 2a) being centred at 75, 155 and 227 nHz respectively, the latter two being the second and third harmonics of peak A. The subsidiary peaks around A, that is, A' and A'', are consistent with the power spectrum of a periodic time series truncated by the 1,185-day observation time interval. The full width of the primary peak A (~ 10 nHz) is somewhat wider than that expected for a 'pure' sine-wave time series truncated at 1,185 days (T) which would be $2/4T = 4.9$ nHz (ref. 6). The other peaks are not identified. Although it is not shown in Fig. 2a, we note that there is no significant power at a frequency of 429 nHz corresponding to the 27-day period of the solar rotation.

A detailed study of the time distribution of the flares and their active regions shows that two regions AR 3317 and AR 3804, were particularly prolific, producing 7 and 11 flares in 1981 and 1982, respectively. Each region produced their flares within a short time span (~ 10 days) and the time interval between the two regions was ~ 10 months. Removal of these 18 events from the time series resulted in a power spectrum which still had the most pronounced peak at 75 nHz, but with its peak power reduced by a factor of 1.5. Therefore, the events of these regions are not the sole cause of the strong peak in the power spectrum at 75 nHz. We have also calculated the power spectral density (PSD) using an independent fast Fourier-transform program⁶ on the same time series. In this case, all the strong features of Fig. 2a are reproduced and at the same frequencies. To estimate the significance level of the peak at 75 nHz, whose formal probability is $>6\sigma$ (ref. 7), four time series were run by this program, replacing the 139 observed events with an equal number of random ones within the same 1,185-day intervals. The PSD from random peaks in these test cases never exceeded

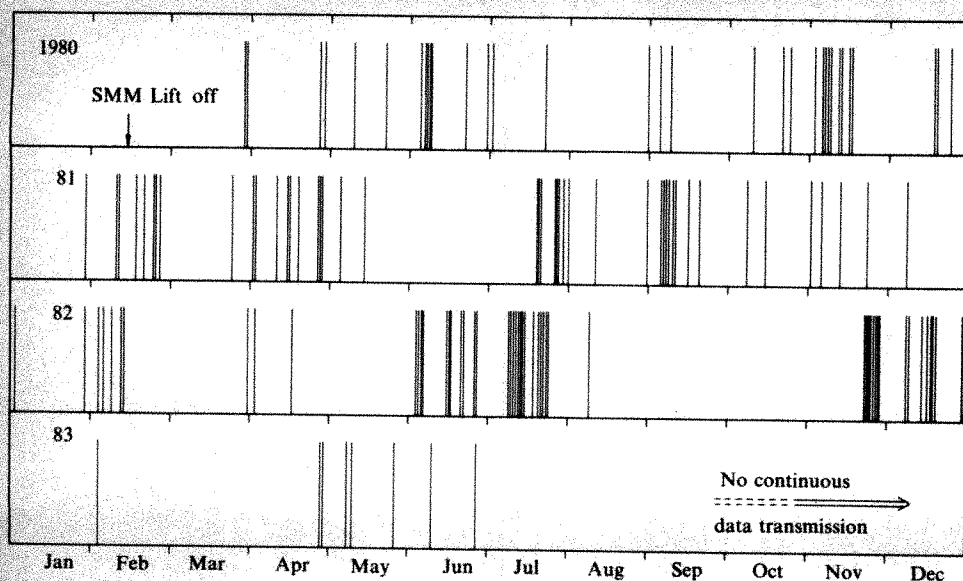


Fig. 1 Energetic solar flare events with photon emission of energy >300 keV are illustrated by short vertical lines, plotted against time for successive years of observation after the GRS began to operate.

$\sim 15\%$ of that for the real peak at 75 nHz, shown in Fig. 2a. This result confirms our formal estimate of the significance of the real peak: it is evident that this peak does not occur by chance and represents a strong 154-day recurrence tendency for energetic photon flares (>300 keV) during the time period of observations.

As already mentioned, we have to work with an incomplete data sample, because about half of the flares cannot be observed owing to the orbit of the SMM satellite. However, the data gaps introduced by the occultation and the close passages of the South Atlantic anomaly are of such a high frequency (170 μ Hz and 11.6 μ Hz, respectively) that they cannot be the cause of any artefacts around the main peak in Fig. 2a. The precessional motion of the orbit, which shows up as a slight change in the instrumental background ($\pm 10\%$) around 300 keV with a period of 48 days (~ 240 nHz), has a negligible influence on the detectability of flares. Finally, a Fourier analysis of 70 cosmic γ -ray bursts observed by GRS as a by-product has revealed no spectral feature around 75 nHz.

If we fold the flare-event times with the apparent period of 154 days, starting on 1980 May 26, and using a bin size of 15.4 days, we obtain the phase histogram shown in Fig. 3. Out of a total of 139 events, 48 (35%) occur within one 15.4-day bin. The broken line indicates the number of active regions which generated the flares in the respective time bin. To test whether the apparent nonuniformity of the latter distribution is statistically significant, we performed χ^2 -analysis. With nine degrees of freedom we obtain $\chi^2 \approx 42$, which corresponds to a probability of 10^{-5} that the time distribution of active regions was caused by chance.

Note also that a similar spectral feature at 75 nHz appears when a Fourier analysis is performed on all Hard X-Ray Burst Spectrometer (HXRBS) events, for which the counting rate was $>10^3$ s $^{-1}$, for photons >25 keV (ref. 8). In this case the total number of flares was 426 and they occurred in a time period overlapping that used in the present analysis and of a similar duration—1,342 as opposed to 1,185 days. As HXRBS is an experiment on the SMM satellite, this data sample also suffers from the fact that the Sun is visible only about 50% of the time. We have therefore analysed flares detected in soft X-rays by the GOES satellites with a GOES class of $\geq M 2.5$ registered during the same time interval³. Figure 2b shows the power spectrum for these 532 events. A prominent peak is seen at 76 nHz, corresponding to a period of 152 days. The difference in the period between the GRS events and the GOES events is insignificant if we take into account the width of the peaks. If we fold the GOES flare event times with the period of 152 days, 168 events (or 32% of the total sample) fall in a single phase bin of 15.2 days. Finally, we removed the 94 GRS flares from

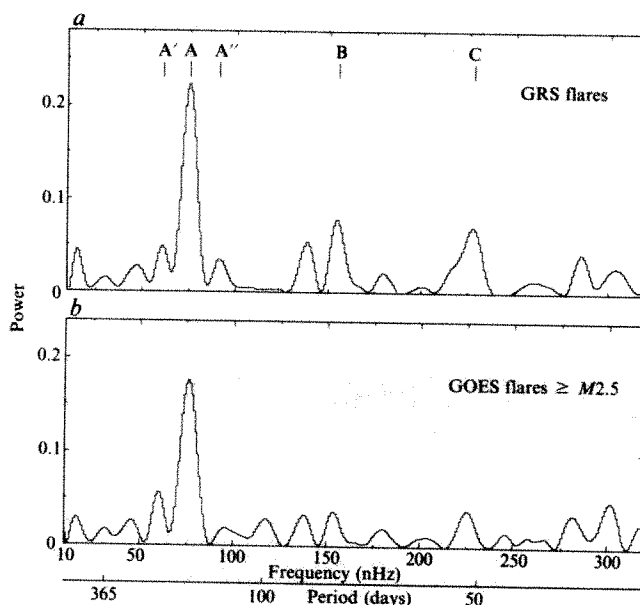


Fig. 2 a, Power spectrum of the time series shown in Fig. 1 (139 events). The individual peaks marked by letters are explained in the text. b, Power spectrum of GOES flares of $\geq M 2.5$ (532 events) which occurred during the same time interval as the GRS flares.

the GOES sample to test whether the regularity of the $\geq M 2.5$ events was caused by the regular occurrence of the GRS flares; but the power in the main peak was reduced by only 6%.

Is this regularity in the occurrence of X-ray flares a feature of cycle 21 which shows up also in other indicators of the solar activity? To check this we have plotted, in the upper part of Fig. 4, the monthly mean sunspot number together with the eight bursts in γ -ray flare activity (phase 0.4–0.5 of Fig. 3). We note also some periodic behaviour in the sunspot number, because the bursts numbered 3, 4, 5, 7 and 8 coincide closely in time with secondary sunspot maxima. For comparison we have also shown the declining phases of cycles 19 and 20 by arbitrarily aligning in time the absolute maxima of each cycle. Cycle 20 is different in appearance from the present one, because it is rather smooth, showing no indication of fluctuations which repeat at 5 months. Cycle 19, on the other hand, is more like cycle 21, because it has well developed secondary maxima, some of which follow in time intervals of ~ 5 months. Dodson and Hedeman⁹ found that increases in solar activity generally occurred with a repetition period of 3–5 rotations. Vitinskii¹⁰ found that the fluctuations in the relative sunspot number range

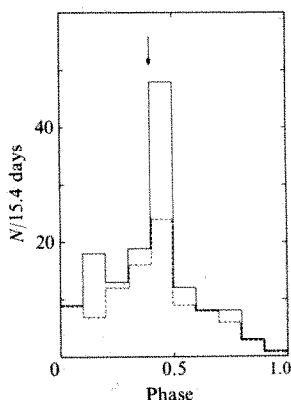


Fig. 3 Number of flares, N , per phase bin, folded by a period of 154 days (full line) and number of active regions contributing flares (dashed line). The absolute times of phase 0.4 (arrow) are: 1980 May 26; 1980 October 27; 1981 March 30; 1981 September 1; 1982 February 2; 1982 July 6; 1982 December 8; 1983 May 12.

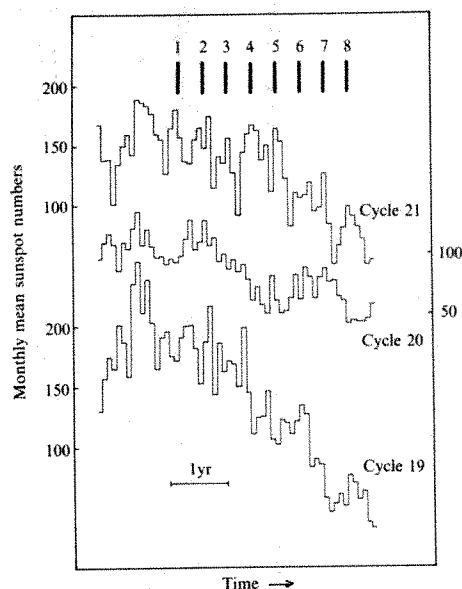


Fig. 4 Monthly mean unsmoothed sunspot number during the declining phases of cycles 19–21 (ref. 4). The cycles were aligned arbitrarily in time with their absolute maxima. The bars at the top of the figure mark the times with respect to cycle 21 when the eight bursts of high-energy flare activity occurred.

in time from 3 months to 1 yr, with an average duration of 5 months. The most extensive analysis of the monthly mean sunspot number was carried out by Wolff¹¹ who searched for significant frequencies using a 230-yr data base from 1749 to 1979 and found a set ranging from 15 to 180 nHz. He interprets these as the beat frequencies of the rotation rate of g-modes. (The g-modes are thought to be oscillations of the solar interior.) The most prominent period of <200 days found by Wolff is 155.4 days (74.49 nHz), which is close to what we have found for energetic solar flares.

Thus there is clear and compelling evidence for a 154-day regularity or periodicity in the occurrence of energetic flares. This effect was first recognized and measured for flares producing emissions of >300 keV and has been confirmed for flares producing soft X rays. The latter data set was obtained from multiple satellites having ~100% solar coverage and hence removes the possibility of any orbital selection effects. This regularity is not a minor effect involving only a few flares but, in fact, involves >30% of all the flares observed. The nature of this 154-day period is not yet understood but may have its origin in deeper layers of the Sun. If so, a better understanding will not only provide information on solar flare production but will

be an important probe of subphotospheric phenomena. It will be interesting to see whether the recurrence period observed for energetic photon flares (>300 keV) persists throughout the remainder of the SMM post-repair mission.

E.R. thanks G. Lichti for valuable discussions. This work was partly supported by Bundesministerium für Forschung und Technologie under 010K017-ZA/WS/WRK 0275:4 and by NASA and the US Air Force under contracts NAS5-23761 and S 70926 A.

Received 31 July; accepted 3 October 1984.

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Magnetic field amplification in the solar nebula through interaction with the T-Tauri wind

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As carbonaceous chondrites are the least thermally-evolved and hence the most primitive of the meteorites, their residual magnetization can, in principle, be used to estimate the intensity of the magnetic field in the primordial solar nebula, which varies between 0.2 and 1 G (refs 1–4), and could be as high as 2–3 G (ref. 4). The presence of palaeomagnetic fields of such magnitude is of importance in reconstructing the early history of the Solar System and of planetary formation. Levy and Sonnet⁵, for example, have stressed this point in comparing the respective merits of four alternative sources of the primordial magnetic field. They claim only two of these are possible: (1) a large solar magnetic field spread into the solar nebula; (2) a hydromagnetic dynamo field generated in the solar nebula itself. We show here that there is in fact a further possibility that fits the requirements for strong magnetic field generation and energetic particle irradiation of the grains⁶: magnetic field enhancement at the point of stagnation between the solar nebula and the intense solar outflows. This mechanism, which involves the interaction of the T-Tauri wind with the solar nebula, is straightforward and is supported by results from recent space research.

We argue basically that during the brief period ($\approx 10^5$ – 10^6 yr) of the T-Tauri phase of the proto-Sun, the solar nebula was being swept up by an intense solar wind with a mass-loss rate of $dM/dt \approx 10^{-7}$ – $10^{-6} M_{\odot} \text{ yr}^{-1}$ (refs 7, 8). This was accompanied by strong flare activities^{7,9} which contributed to the grain irradiation effect. However, the penetration of solar particles should at first be limited only to the inner edge and the boundary layers of the solar nebula (Fig. 1). This scenario is compatible with Wetherill's idea¹⁰ that, because of the blocking effect, the condensed grains forming Venus should be subjected to much more severe effects of primordial solar wind by implantation (^{20}Ne and ^{36}Ar , say) than those later forming the Earth. However, we require that the 'working surface' or the stagnation point between the T-Tauri wind and the inner edge of the solar nebula should progress outward to reach the condensation zone of the carbonaceous chondrites (that is, a solar distance $\approx 3 \text{ AU}$).

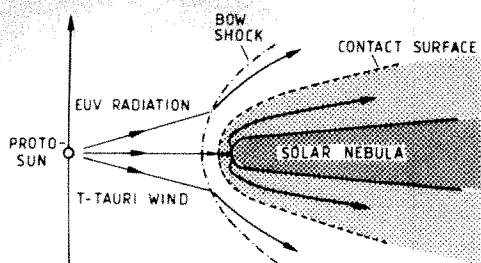


Fig. 1 A schematic view (not to scale) of the interaction process between the solar nebula and the T-Tauri wind of the early Sun. We postulated that, at the stagnation point between the solar wind and the solar nebula, the interplanetary magnetic field would be amplified to 0.1–2 G. Further leakage of the draped fields into the solar nebula would lead to magnetization of the condensed grains. EUV = extreme ultraviolet.

It is not known whether the solar nebula was clear of gas during the T-Tauri phase. In any event, even if there were little gas in the dust disk, the sputtering effect of the flare particles should be effective in generating a gas cloud. The subsequent ionization effect of the solar UV radiation and the charge-exchange process between the T-Tauri wind and the neutral gas will lead to a situation similar to that of solar wind interaction with comets, or Venus, in which the interplanetary magnetic fields are compressed (or draped) ahead of the ionospheres of these planetary bodies¹¹.

Interplanetary magnetic fields (flux $B_{\infty} \approx 1 \times 10^{-4}$ G), as demonstrated by the Pioneer Venus observations¹², are piled ahead of the contact surface separating the ionosphere of Venus and the shocked solar wind. The amplification effect can be approximated by equating the magnetic field pressure to the ram pressure of the solar wind. A similar relation has been used to estimate the interplanetary magnetic field piled ahead of a cometary ionosphere¹³, and detailed magnetohydrodynamic calculations^{14–16} have shown that the Lorentz force due to the radius of curvature of the captured field lines will be important, and will lead to further enhancement of the magnetic field. That is, if B_i is the peak field flux at the stagnation point, it could be given by

$$\frac{B_i^2}{8\pi} \approx (2-3)\rho_{\infty} V_{\infty}^2 \quad (1)$$

where ρ_{∞} and V_{∞} are the mass density and speed of the solar wind at large upstream distances, respectively. In the case of the T-Tauri wind, with $dM/dt \approx 10^{-7}$ – $10^{-6} M_{\odot} \text{ yr}^{-1}$, a wind speed (V) = 200 km s⁻¹, and a solar distance of ~3 AU from the proto-Sun, the application of equation (1) indicates that the magnetic fields in the vicinity of the working edge of the solar nebula could be amplified to ~0.2–0.6 G even with a small seed field in the T-Tauri wind.

Note that the above description of magnetic field amplification in the vicinity of the stagnation point depends very much on the validity of treating the entire interaction process as continuous. It is possible also that kinetic effects might have been important in the actual situation, as argued previously for the case of comet-solar wind interactions¹⁷. But at least the observations at Venus, where these effects could certainly be said to be important in the solar wind interaction process, have indicated that equation (1) is not inappropriate.

In this connection, there is also an important difference between the azimuthally symmetric disk geometry of the solar nebula and those of comets and Venus. Most significantly, in the case of the solar nebula, the draped fields would not be moved away from the flanks by convection. In other words, whereas the stagnation points with enhanced magnetic fields are only localized regions in the vicinity of the hemispherical nose-cones of comets and Venus, for the solar nebula (if axisymmetrically shaped) it could form a belt-like structure extending 360° around the Sun. Without sideways-slippage, all the magnetic flux may tend to accumulate at the front. From this point of view, the maximum value of the draped fields could be

increased further beyond the prediction of equation (1). Whether it could reach 2–3 G, as obtained by setting the magnetic pressure equal to a thermal pressure of 10^{-6} bar in a limited region near the contact surface, must be resolved by future quantitative computations. Note that the continuous build-up of the magnetic fields at the front could be balanced only by continuous leakage into the inner region of the solar nebula or reconnection near the stagnation point. Both processes could take place: however, the leakage effect (by interchange instability or ambipolar diffusion) is more interesting as it allows an extensive portion of the solar nebula to be permeated by the draped magnetic fields with a magnitude of ~0.2 G. These fields would eventually be dissipated by magnetic diffusion or, once again, by magnetic reconnection at the equatorial current sheet.

We have extended the magnetic field amplification effect which has been observed in the solar wind-Venus interaction, and which is expected to occur in the solar wind-comet interaction, to the interaction of the dust and gas components of the primordial solar nebula with the T-Tauri wind of the proto-Sun. Using this extrapolation, strong magnetic fields (at least ≥ 0.1 G) could be generated at the stagnation point of the solar nebula and could even penetrate through the solar nebula to form a thin current sheet at the equatorial plane separating the two regions of opposite magnetic polarity. Whether or not a maximum field strength of 2–3 G could be generated, as inferred from the large permanent magnetization of the Allende meteorite, remains unclear. In any event, we believe that our proposed mechanism is promising compared with the alternatives reviewed by Levy and Sonett⁵ and they should be investigated further. We note that the present model may be considered as an important variant of the one involving spreading of the solar magnetic field into the solar nebula (proposal (1))⁵. We expect in fact that a hydromagnetic dynamo field might be generated in the solar nebula (proposal (2))⁶ concurrently with the field-draping mechanism proposed here. However, the dynamo mechanism depends critically on the state of turbulence of the solar nebula, whereas the T-Tauri wind interaction scenario is relatively insensitive to the particular assumptions of turbulent velocity and scale-length of the turbulent eddies. Perhaps another point of interest is the azimuthal motion of the ionized matter in the vicinity of the contact point. In an idealized picture, such 'winding' of the draped fields should act to produce a toroidal component of the amplified field: either the draped field could be further enhanced by this process, or it could introduce complicated effects relating to the stability of the system which could be treated only in detailed numerical models. This particular issue, together with the questions of reconnection and leakage of magnetic fields, must await quantitative studies. Finally, in this connection, we note there is uncertainty about the timing between the T-Tauri phase of the proto-Sun and the condensation of the carbonaceous chondrites. However, the importance of the quasi-cometary interaction process as an element in the magnetohydrodynamics of the early solar nebula, as depicted here, is quite independent of these considerations.

Received 29 May; accepted 4 October 1984.

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Observation of a thin layer of material in the upper stratosphere

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In this letter we report the results of laser radar measurements at Aberystwyth ($52^{\circ}25' \text{ N}$, $4^{\circ}04' \text{ W}$) which revealed the presence of a discrete layer of atmospheric material of about 0.5 km thickness near 40 km for at least 3 h during the night of 4–5 July 1983. Wind data indicate that it was moving from the east and had an east–west extent of at least 170 km. We estimate that the layer had a mass content in excess of 100 kg for each degree of latitude width. This layer does not appear to be due to a natural terrestrial source or extraterrestrial material, and it seems likely that it was a result of pollution at upper stratospheric heights.

The observational system makes use of a Nd/Yag laser transmitting 300 mJ of energy at 530 nm in 15-ns pulses at a repetition rate of 10 Hz. The receiver includes a 1-m newtonian telescope fitted with a suitable interference filter and operated in a photon counting mode, the recording channels providing a height resolution of 60 m. The variations with height over the interval 36–42 km of the range-corrected backscattered signals recorded in successive 15-min periods between 22.05 UT on 4 July and 02.20 UT on 5 July 1983, are shown in Fig. 1. For clarity, the data are plotted as running means over 300 m, the standard errors for these means being presented at intervals of 1.5 km. The results for the first five 15-min periods show smooth height variations consistent with Rayleigh scattering. A small perturbation is shown near 39.5 km in the height variation shown for the sixth period, 23.20–23.35 UT on 4 July, and this feature becomes more pronounced with time, increasing to maximum amplitude in the fourteenth and fifteenth periods, 01.20–01.35 and 01.35–01.50 UT on 5 July, after which it seems to diminish. Unfortunately, observations were discontinued at the end of the seventeenth period at 02.20 UT. Measurements have been carried out on most clear nights since November 1982 with height resolutions of 300 m or better but no other example of such a thin layer has been observed. Note, however, that the greater degree of variability in the height variation of Rayleigh scattered signals observed on winter nights might obscure such a feature.

The change in magnitude and form of the perturbation with time is indicated in Fig. 2. This shows the mean data for periods 6–8 (23.20–00.05 UT), 9–12 (00.05–00.50 UT), 12–14 (00.50–01.35 UT) and 15–17 (01.35–02.20 UT) expressed in terms of the ratios R of the mean data for corresponding heights in the earlier periods 1–5 (22.05–23.20 UT). To provide representative measures of the peak height and thickness, running means over 120 m height are adopted. The values of $R-1$ represent the excess backscatter relative to the Rayleigh scatter from molecules at the corresponding height. The peak value approaches 10% for the periods 12–14. Based on the molecular densities from an atmospheric model for 45° N in midsummer¹, the integrated backscatter for these periods is estimated at $3.15 \pm 0.30 \times 10^{-7} \text{ sr}^{-1}$. If the scattering particles were composed of a sulphuric acid–water solution, as found at lower stratospheric heights, this value corresponds to an integrated mass of $5 \times 10^{-9} \text{ kg m}^{-2}$ (ref. 2).

Observations of dust layers in the upper stratosphere and lower mesosphere have previously been made from orbiting spacecraft³, aircraft⁴, balloons⁵ and the ground^{6,7}. Most of these observations related to intermittent layers of thicknesses 10 km or greater at heights near 50 km or above. However, Clemesha and Nakamura⁷ reported laser radar observations of a layer of thickness 4 km, which dropped from 47 to 44 km within a day, and excess scattering over a range of heights during three subsequent months. They associated their observations with the influx of extraterrestrial material.

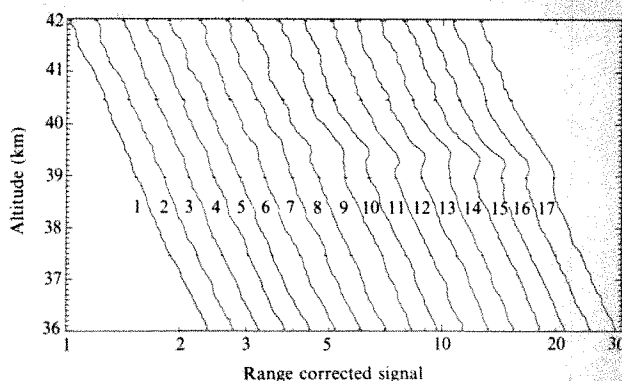


Fig. 1 Variations with height of the range-corrected signals at vertical incidence measured in 15-min intervals during 22.05 UT on 4 July to 02.20 UT on 5 July 1983; the plots for successive intervals are offset along the horizontal axis. Standard errors are shown at intervals of 1.5 km.

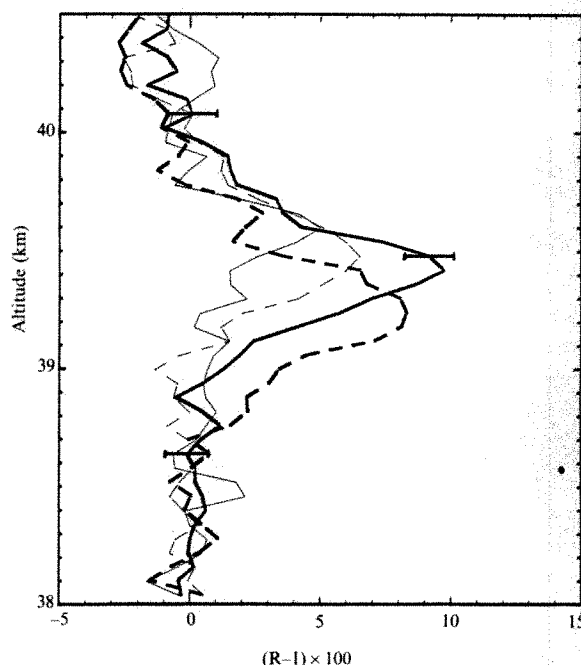


Fig. 2 Variations of the excess backscatter arising from the perturbation as deduced for four 45-min periods: — 23.20–00.05 UT; --- 00.05–00.50 UT; —·— 00.50–01.35 UT; ··· 01.35–02.20 UT. R represents the ratio of the mean backscatter signal in these periods to the corresponding mean signals prior to the perturbation. Sample standard errors are shown.

The presence of aerosols, of variable concentrations, is a permanent feature of the lower stratosphere. However, measurements using several techniques, both ground-based and space-borne, have demonstrated that the material is generally confined to heights below 30 km (ref. 8). The violent eruption of the El Chichón volcano in Mexico in March–April 1982 did produce aerosols at heights extending up to 35 km during the first few months following the eruption^{9,10}, and observations at Aberystwyth recorded the presence of intermittent layers up to 34 km until the end of 1982. Subsequently, the enhanced aerosol concentrations were generally confined to heights below 30 km (ref. 11) and by June 1983 there was no evidence of aerosols at heights above 30 km. However, it is of interest to consider the El Chichón eruption as a possible source of the observed layer.

To be detected by scattering at 530 nm, the particles probably have radii of at least 0.1 μm . From the subsidence speeds presented by Kasten¹², sulphuric acid or silicate particles of radii 0.1 μm and densities 1.6 g cm^{-3} and 2.5 g cm^{-3} , respectively, injected to heights up to 50 km at the time of the eruption would have fallen to the height of the observed layer

within about 3 months and subsequently merged with the main aerosol layer below 30 km within about a year. If composed of the very low density silicates or ash also reported in larger particles near 20-km altitudes and below^{13,14}, they could remain for much longer times under subsidence. However, it is unlikely that such a thin layer could have survived more general dynamical processes; vertical turbulent diffusion, for instance, would double the width of the layer in about 1 day, even with the lowest value of coefficient currently expected ($1 \text{ m}^2 \text{ s}^{-1}$)¹⁵. If the particles represented, instead, sulphuric acid droplets produced from the oxidation of SO_2 known to be injected into the stratosphere at the time of the eruption^{16,17}, it can be shown that for an OH concentration of $\sim 3 \times 10^7 \text{ cm}^{-3}$ (ref. 18), the lifetime of the SO_2 against oxidation would have been about four days. Coagulation and growth of the sulphuric acid particles could then have occurred to produce the particles observed. However, at the temperature of 260 K existing near 40 km (deduced from the height distribution of molecular densities provided by the Rayleigh scatter measurements), evaporation should have occurred more rapidly unless the partial pressure of ambient sulphuric acid had been substantially increased. On the basis of these arguments, it seems unlikely that the observed layer could be associated with the El Chichón eruption.

If the observed layer was associated, instead, with cosmic dust particles burning out in the atmosphere, the observed thickness could have arisen from the compression of a thicker layer produced at a greater height, because of the difference in fall velocity between the top and bottom, rather than being produced *in situ*. It is found that for the heights of interest the fall velocities of particles of radii 0.003 to $10 \mu\text{m}$ as given by Kasten¹² vary with height as the inverse of the pressure. From this result the locations of initial layers of different thicknesses which could have produced the observed layer after subsidence can be calculated. For the layer observed in periods 12–14 (00.50–01.35 UT on 5 July) (Fig. 2), the half-peak points occur at 39.2 and 39.6 km. From the pressures given in the atmospheric model for midsummer at 45°N (ref. 1), it can be shown that an initial layer of thickness 5 km would have had a base at 57.3 km, almost independently of the particle radius, and thicker layers would have had slightly higher bases. However, the creation of the thin layer by subsidence implies that the particles would have a very small range of sizes. Thus for particles falling from 57.3 km to a final layer between 39.2 and 39.6 km the range of sizes can be shown to be $< 1\%$. This restriction in size need not be applicable in the case of $0.1 \mu\text{m}$ particles, if these represent the largest particles in the distribution, because smaller particles could have been distributed above the observed layer and would not have been detected by the laser radar equipment. For particles of radii $0.1 \mu\text{m}$ and densities 3.5 g cm^{-3} (ref. 19), the fall time for an initial layer of 5-km thickness with a base at 57.3 km would have been 58 days. However, the major part of this time would be spent near the final height and it is unlikely that the thin layer could have persisted for such a time in the presence of turbulent diffusion.

An upper limit for the size of the subsiding particles can be derived from the reduction in height of the maxima with time in Figs 1 and 2. The apparent downward velocity deduced is $\sim 5 \text{ cm s}^{-1}$, which for a density of 3.5 g cm^{-3} corresponds to a radius of $2.9 \mu\text{m}$ (ref. 12). The subsidence times for the layer having an initial base at 57.3 km would then be $\sim 11 \text{ h}$, and the effect of turbulent diffusion, as considered previously, would not be significant. However, in this case, the restriction on the range of sizes of the subsiding particles would apply. It would be unreasonable to associate these particles of a single size with a meteoric event.

In view of the difficulties encountered in relating the observed layer to a natural terrestrial origin or to extraterrestrial material, consideration should be given to its creation by man-made agencies. It would then be of interest to examine some of the features of this stratospheric pollution. Estimates of geostrophic wind velocities from the Stratospheric Sounder Units on NOAA satellites by the Meteorological Office show predominantly east-

west winds at stratospheric heights at noon on 4 and 5 July 1983, with a mean value at the level of the cloud of $\sim 16 \text{ m s}^{-1}$. The cloud, therefore, moved from the east with that velocity and had an east-west extent of at least 170 km, based on the 3 h for which it was observed, and this implies a mass content in excess of 100 kg for each degree of latitude width.

We acknowledge valuable discussions with Drs A. D. Maude and G. Vaughan, and the receipt of stratospheric wind data from Dr A. O'Neill of the Meteorological Office, Bracknell.

Received 27 July; accepted 11 October 1984.

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Displacement of the Moho by the Outer Isles thrust shown by seismic modelling

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Deep seismic data (Fig. 1) from the Caledonides, off the north-west coast of Scotland, collected (1981–82) by the British Institutions Reflection Profiling Syndicate (BIRPS), show evidence that Caledonian thrusts have been reactivated as Mesozoic normal faults^{1,2}. The best example is the Outer Isles (OI) thrust, which is mapped on land as a compressional fault, and is revealed by these data to be overlain offshore by a thick, wedge-shaped Mesozoic rift basin¹, known as the North Lewis (NL) basin. Here we have used synthetic seismic sections to identify distortions of the OI thrust reflection due to the NL basin. Models, which compensate for the effects of this distortion, show that the OI thrust has a steeper angle of dip than has been predicted previously assuming no distortion, causing the OI thrust to project into a 2–3 km offset of the Moho and hence to penetrate the entire crust. Our data, suggesting that shear strains affecting the lower crust may be localized in character, have adverse implications for rheological models which predict, to the contrary, that the lower crust is a region where strain is distributed.

Figure 2 shows the unmigrated fault reflection of the OI thrust dipping $20\text{--}22^\circ$ to the east. All angles given here were calculated using a 6 km s^{-1} velocity for converting time to depth. A second, deeper reflection from the Flannan thrust¹ dips at approximately the same angle as the OI thrust reflection. On the basis of other BIRPS data, the Flannan thrust has been interpreted to be a normal fault displacing the Moho to the west of the section shown in Fig. 2. The Flannan thrust extends upwards from within the mantle (15 s two-way travel time, or about 45 km depth) into the lower to middle crust, but does not appear to reach the surface. The OI thrust, conversely, is mapped at the surface, and can be traced on the BIRPS data to a depth of $\sim 20 \text{ km}$, or 6.8 s two-way travel time. The observation that

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reflections from both the OI thrust and the Flannan thrust apparently terminate in the lower crust led Matthews and Hirn³ to propose a model in which the two thrusts merge into a lower-crustal ductile zone, with neither penetrating the entire crust. They propose a similar model for the Himalayas, where critical-angle and wide-angle reflection data suggest the existence of Moho-displacing faults that cannot be traced to the surface⁴.

The unmigrated time section for the WINCH-1 survey (Fig. 2) shows a down-to-the-east displacement of the Moho reflection below the OI thrust, at a distance of ~33 km. The Moho reflection drops abruptly from 7.5 s two-way travel time to below 8 s. The identification of this reflection with the Moho is supported by results from a nearby refraction profile⁵. At first glance, the Moho displacement does not appear to be related to the OI thrust, as conventional time migration fails to steepen the fault plane reflection sufficiently for it to intersect the Moho at the point of displacement. Migration of the 20–22° reflection steepens it by only 1–2°. Here we use seismic modelling to show that velocity pull-down effects from the overlying NL basin mask the connection between the OI thrust and the Moho displacement; the OI thrust actually extends completely through the crust and displaces the Moho.

Velocity pull-down—the travel-time delay of reflections from structures underlying low-velocity material—can be seen on the prominent Moho reflections present in the data. On WINCH-1 (Fig. 2), these velocity effects are seen as a proportional increase in east-west travel time to the Moho as the overlying NL basin thickens. The Moho reflection is at 7.5 s two-way travel time east of the basin, and increases steadily to 8.0 s at the western edge of the data range, where the sediments are thickest. Dipping events underlying the basins, such as the OI thrust, also undergo velocity pull-down, but the distortion is not as readily apparent as the pull-down of the horizontal Moho reflection. Time migration, using a constant velocity for the overlying material, does not take this velocity-related distortion into account and will not correct for the pull-down effect.

Figure 3 shows a simple geological model for WINCH-1, developed to evaluate the geometry of the OI thrust more accurately. The model differs from the interpretations of Brewer *et al.*² and Matthews and Hirn³ in that the OI thrust dips at 29° rather than 20–25°, and extends completely through the crust to displace the Moho, rather than ending in the lower crust. Interval velocities calculated from stacking velocities obtained during original processing⁶ were smoothed and used to calculate travel times. Normal incidence, or zero-offset ray tracing, and convolution with a synthetic wavelet, produced synthetic time sections. A model in which the OI thrust dips at 29° and intersects the Moho displacement (Fig. 3) produces a synthetic seismic section (Fig. 4) which successfully matches the data. Velocity effects produced by the NL basin act to lessen the angle of the reflection from the OI thrust by 4–6° on the time section, compared with the angle it would have in a homogeneous crust. Models with velocity structures differing by up to +0.7–0.8 km s⁻¹ for the basin layers and +0.3 km s⁻¹ for the crust do not change this result significantly. However, models in which the OI thrust dips at only 20–22° do not produce a synthetic section which matches the data, unless the entire basin is composed of sediments with velocities of ~6 km s⁻¹. Sedimentary velocities of this magnitude do not match the interval velocities calculated from the data, nor do they produce the pull-down on the Moho observed in the seismic data.

The modelling results do not significantly change the geological interpretation of the Flannan thrust. However, the dip of the Flannan thrust reflection has also been decreased by velocity pull-down, and models with a dip for the Flannan thrust of 32–34° show best agreement with the original data. This thrust appears to have the same dip on the time section as the intracrustal OI thrust but, because it is most clearly seen in the upper mantle, where the seismic velocities are higher (8 as opposed to 6 km s⁻¹), a steeper dip is needed to produce a reflection parallel to the OI thrust reflection.

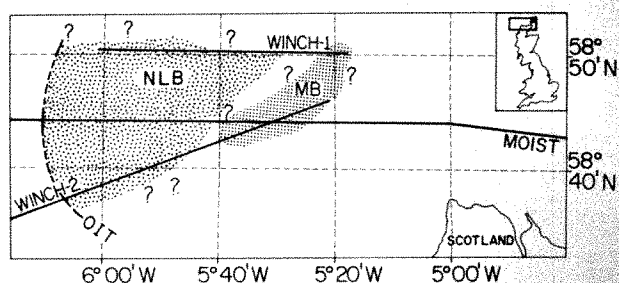


Fig. 1 Location map showing WINCH-1, MOIST and WINCH-2. Carets show the inferred extent of North Lewis basin (NLB). Note that the basin narrows to the south as it crosses WINCH-2. Dots show location of the Minch basin. The Minch basin is more fully developed on WINCH-2 and MOIST than on WINCH-1, which crosses only the shallow, northernmost portion of the basin.

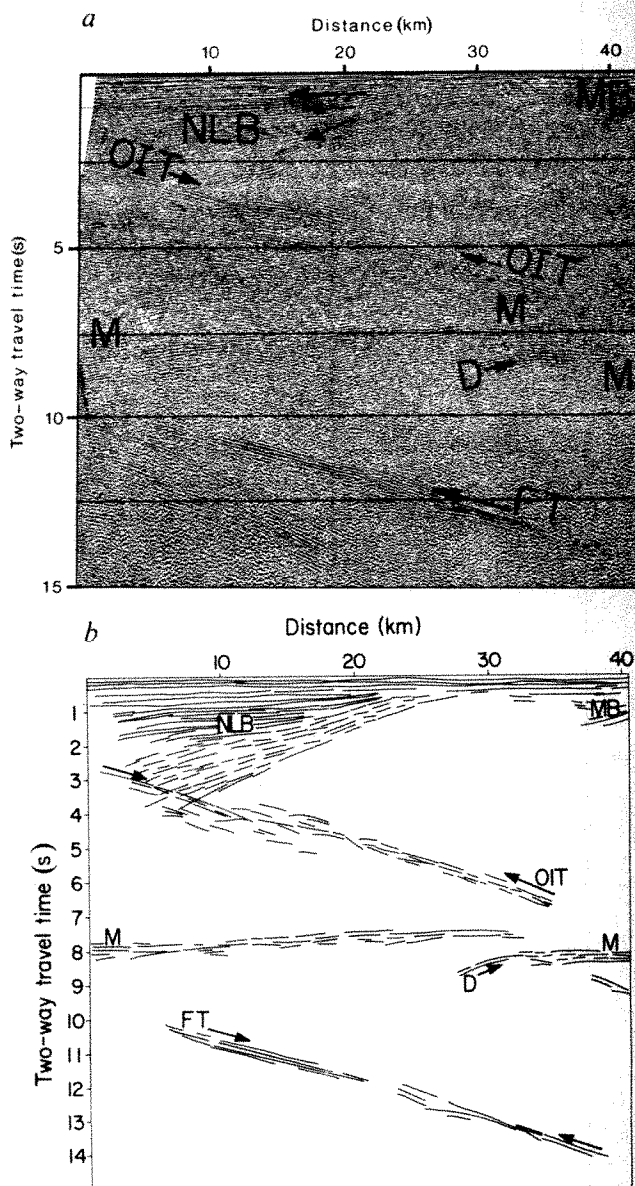


Fig. 2 a, WINCH-1, unmigrated; b, line drawing of WINCH-1. FT, Flannan thrust; OIT, Outer Isles thrust; MB, Minch basin; NLB, North Lewis basin; M, Moho. Note a 600–700 ms down-to-the-east displacement of the Moho at 33 km. The OI thrust reflection dips at 20–22°. The shallow, overlying Minch basin contributes slightly to the displacement of the Moho by adding a negligible amount of velocity pull-down. The Minch basin contributes ~500 ms of pull-down on the Moho seen on MOIST and WINCH-2, where the basin is much better developed. Event D fits diffraction curves for crustal velocities. The presence of a diffraction suggests that the displacement is real rather than a velocity effect.

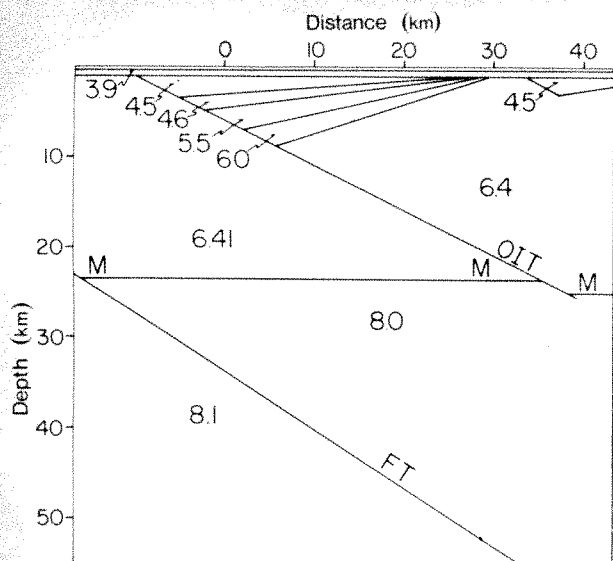


Fig. 3 Geological model for the OI thrust in which the fault actually dips 29° and displaces the Moho at 33 km. The Flannan thrust dips at 33°. Velocities were smoothed from interval velocities obtained by BIRPS during processing of the data⁶, and are in km s^{-1} . Although these velocities are relatively high, the sediments comprising the NL basin are believed to be mainly Palaeozoic (D. K. Smythe, personal communication).

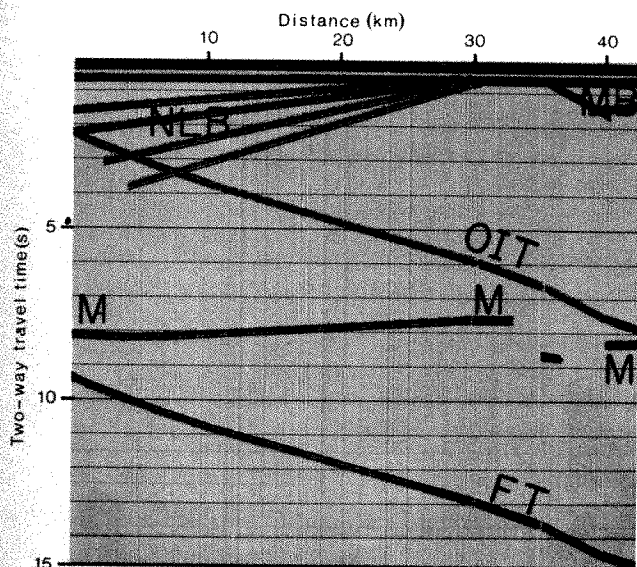


Fig. 4 Synthetic seismic section for the model in Fig. 2. Although the OI and Flannan fault planes dip at 29° and 33°, respectively, their reflections dip only 20–22° on the seismic section. If the NL basin did not overlie the OI thrust, the reflection from the fault would dip at 26° on the unmigrated seismic section.

Three-dimensional control of these structures is provided by MOIST, a previous BIRPS survey¹, parallel to WINCH-1, but extending much further east, and by WINCH-2, which intersects MOIST in the Minch basin (Fig. 1). The OI thrust reflection dips at 20–22° on MOIST and 24–25° on WINCH-2. The observation that the NL basin is less well developed on WINCH-2 agrees well with its steeper dip on this line. All three seismic lines are as close as possible to dip lines, based on unpublished shallow data (D. K. Smythe, personal communication). Similar modelling suggests that a Moho displacement is associated with the OI thrust on both MOIST and WINCH-2. However, the displacement is obscured on MOIST and WINCH-2 by velocity pull-down associated with the Minch basin, which is much thicker on these lines than on WINCH-1. The OI and Flannan thrusts are most successfully modelled on MOIST by dips of

29° and 32–33°, respectively, as for the parallel WINCH-1.

The OI thrust reflection ends at a much shallower depth on MOIST than on WINCH-1, where it can be traced almost completely through the crust. The termination of the OI thrust reflection corresponds in each case to the beginning of the Minch basin at the surface, suggesting that the absence of a reflection from the OI thrust in the lower crust is owing to complications associated with the Minch basin sediments, rather than the termination of the fault at different crustal levels on the two lines.

Brewer and Smythe^{7,8} have proposed that the OI thrust displaces the Moho on MOIST, based on the intersection of the Moho with an extrapolation of the migrated OI thrust fault-plane reflection. However, because the apparent dip of the migrated fault reflection (22–23°) differs from the dip of the fault plane by 6–7°, through pull-down, these extrapolations lead to an intersection ~20 km east of the displacement identified here. The apparent Moho displacements seen on MOIST at these other locations are caused by velocity pull-down from overlying basins; it is simply coincidental that the distorted OI thrust reflection projects into one of these apparent displacements.

In conclusion, the new modelling indicates that the OI thrust does not end in a ductile zone in the lower crust, but continues completely through the crust into the uppermost mantle. Although this result does not preclude the existence of a ductile lower crust, it does demonstrate that localized zones of deformation are likely to exist at these depths.

The synthetic seismic sections presented here were calculated using the Advanced Interpretive Modelling System (AIMS, Geoquest International) on the Megaseis (Seiscom Delta) seismic computing system operated by the Consortium for Continental Reflection Profiling at Cornell. The research was supported by NSF grants EAR82-12445 and EAR83-13378. These results were presented at the International Symposium on the Deep Structure of the Continental Crust at Cornell University, 26–28 June 1984. I thank Simon Klemperer, David Smythe, Larry Brown and Michael Warner for comments. Contribution 805 of Department of Geological Sciences, Cornell University.

Received 23 July; accepted 28 September 1984.

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Palaeoclimate of Baffin Bay from 300,000-year record of foraminifera, dinoflagellates and pollen

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Several different models^{1–5} have attempted to relate Quaternary marine and terrestrial records of ice-sheet growth in the North Atlantic region. The models are derived from either discontinuous terrestrial records, including marine fossils in raised shorelines^{1,2}, or from deep-sea calcareous microfossils^{3,4}. This study correlates Arctic marine and terrestrial data directly by comparing $\delta^{18}\text{O}$, foraminiferal, dinoflagellate and pollen records from a core taken in Baffin Bay. The core contains an almost continuous sequence of sediments deposited during isotope stages 1–10. Subarctic foraminifera and Atlantic dinoflagellates in early isotope stages

2, 4, 6 and 8 show that subarctic water entered southern Baffin Bay before the glacial maxima. Boreal-subarctic pollen suggest that Atlantic air flowed into Baffin Bay during the ice sheet growth phases. These data support models which postulate that open water in the Labrador Sea had an important role in supplying moisture to Laurentide and Greenland ice sheets.

Data for this study of ocean-atmosphere interaction during the late Quaternary were obtained from core 77-027-017 taken from a water depth of 935 m on the northern slope of Davis Strait at 66° 54.1'N, 58° 17.7' W (Fig. 1). The core includes a thin surface layer of brown diatomaceous mud overlying detrital carbonate-rich gravelly sand and ~10 m of partially bioturbated olive-grey sandy or silty mud. Planktonic and benthic foraminifera were studied in the >63 µm fraction using standard procedures⁶ to provide a biostratigraphical and palaeoceanographic record. Previous studies in Baffin Bay⁷ indicate that calcium carbonate dissolution limits the information content of calcareous microfossils; therefore, pollen and dinoflagellate cysts were also studied in core 017, using extraction methods⁸ suitable for clayey muds. Pollen and spores in marine sediments provide a link between terrestrial and oceanic environments and, off eastern Canada, pollen assemblages show a strong correlation with onshore climatic zones⁸. Most dinoflagellate cysts are spores of planktonic algae and, in marine sediments, they are proxy-indicators of surface water conditions^{9,10}.

The oxygen isotope composition of planktonic foraminifera in core 017 (Fig. 2) shows a typical isotopic record extending into stages 10 as defined by Emiliani¹¹. Calcareous foraminifera are absent from the top of the core because of the present shallow lysocline⁷. Gastropod shells from ~50 cm depth yielded a ¹⁴C age of 10,530 yr BP, confirming the isotope stage 1-2 transition between 60 and 70 cm. From 75 to 300 cm, high $\delta^{18}\text{O}$ values represent isotope stages 2, 3 and 4. At 300 cm, a large (2.5%) shift in $\delta^{18}\text{O}$ marks the top of stage 5. Stage 6 (465-515 cm) is rather compressed. Stage 7 (515-630 cm) has several light isotopic peaks, and a thermoluminescence (TL) age of $281,000 \pm 20\%$ yr BP on silt from 625 to 627.5 cm generally agrees with the age¹¹ of 251,000 yr BP for the base of stage 7. Stages 8 and 9 contain two light peaks. Below 810 cm, the record lies within stage 10.

Although the number and diversity of planktonic and benthic foraminifera in core 017 (Fig. 2) are low, there are cyclical variations in their percentage abundance. Stage 1 contains only arenaceous benthic foraminifera, typically *Textularia earlandi*, *Trochammina inflata*, *T. nana*, *Eggerella advena* and *Reophax arctica*, with abundant diatoms and radiolaria. In the Canadian Arctic, this benthic assemblage is generally confined to areas of cool saline bottom waters¹², and is often associated with carbonate dissolution⁶. Benthic foraminifera in stages 3, 5, 7 and 9 are mostly calcareous species dominated by *Cassidulina reniforme*, *Elphidium excavatum* and *Fursenkoina fusiformis*. This fauna characterizes silty sediments below cold and low-oxygen waters. Planktonics are almost all *Neogloboquadrina pachyderma* (sinistral) with abundant diatoms and radiolaria. Calcareous foraminifera in stages 3, 5, 7 and 9 are largely lost to dissolution⁷. Benthics in stages 2, 4, 6, 8 and 10 are predominantly calcareous, mainly *Islandiella teretis*, *Nonionella labradorica*, *Buccella frigida*, *Astrionion gallowayi* and *E. excavatum*. This fauna presently occupies deep basins with relatively warm bottom water on the eastern Canadian margin¹².

Planktonic foraminifera in core 017 include *N. pachyderma* (sinistral and dextral), *Globigerina quinqueloba* and *G. bulloides*, with up to 15% subarctic species. Modern distributions of subarctic planktonics are confined to warmer (4-10°C) surface waters; beneath the West Greenland Current, *G. quinqueloba* and *G. bulloides* make up to 25% of the planktonic fauna. The presence of subarctic foraminiferal peaks in the early glacial stages of core 017 suggests significant advection of subarctic surface water into northern Labrador Sea at these times (refs 6, 13, and manuscript in preparation). This would occur if the Arctic Island channels were largely blocked by ice, thereby reducing Arctic water outflow to the North Atlantic. Decreased

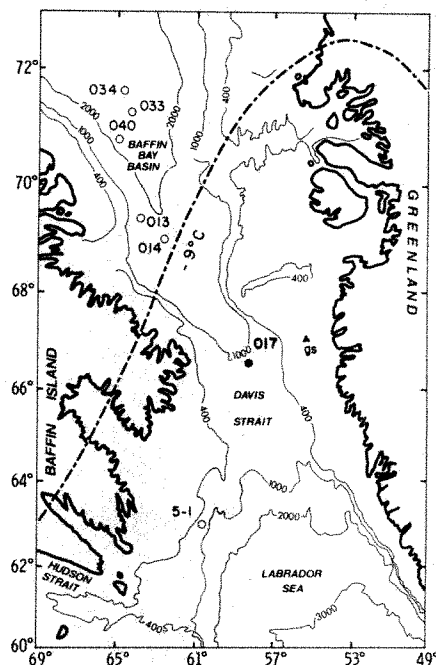


Fig. 1 Bathymetry of Baffin Bay (contours in m) showing the location of core 017 and other cores (ref. 13 and work in preparation) with detailed oxygen isotope, microfossil and/or palynological data; gs, grab sample used for isotopic data at the top of core 017 (see Fig. 2). The -9°C mean annual air isotherm (---) marks the average position of the Atlantic-Arctic air front.

surface runoff and reduced north-east Atlantic inflow would also result in a weaker Baffin Land Current during glacial periods⁶, thus allowing northward penetration of subarctic waters.

Data from core 017 and other cores from Davis Strait^{6,13} show that the interglacial strata are depleted in planktonic foraminifera but rich in siliceous/arenaceous microfossils, whereas the opposite is true for glacial strata. Alternation of predominantly siliceous/arenaceous and calcareous microfossils is typical of areas where carbonate cycles are a function of dissolution cycles⁷. Therefore, acid-resistant palynomorphs were studied in 42 samples taken as closely as possible to the foraminiferal sample intervals.

Figure 3 shows that Quaternary dinoflagellates are common although the numbers generally decrease downcore. The Atlantic dinoflagellates are species which presently have maximum percentage abundances in the temperate North Atlantic¹⁰, particularly beneath the North Atlantic Drift. These species include *Operculodinium centrocarpum*, *Nematosphaeropsis labyrinthea*, *Spiniferites ramosus*, *S. elongatus*, *S. bulloides* and *S. membranaceus*. Their modern distribution in Baffin Bay⁹ is confined to deep-water areas beneath the relatively warm (2-5°C) West Greenland Current. In Core 017, these species occur mainly in the interglacial stages 1, 5 and 7, but they also occur in glacial isotopic stage 2 and in the lower parts of stages 4, 6 and 8. The most common species in core 017 is *Multispinula minuta* which dominates the Canadian Arctic dinocyst assemblage⁹ found in modern sediments below the cold Baffin Land and Labrador Currents. In core 017, however, *M. minuta* dominates only in the glacial stages 6, 10 and upper 8, where it is associated with *Brigantedinium simplex*, *Peridinium faeroense* cysts and a *Leiosphaera* species⁹ which is presently abundant in Arctic channels north of Baffin Bay.

The main features of the dinoflagellate stratigraphy are (1) peaks of Atlantic species which often correspond to decreases in subarctic foraminifera; (2) changes in the ratio of the warm water indicator *O. centrocarpum* to the Arctic indicator *M. minuta*, with peaks of *O. centrocarpum* often inversely related to the subarctic foraminiferal peaks; and (3) large increases in dinoflagellate concentrations which mostly correspond to

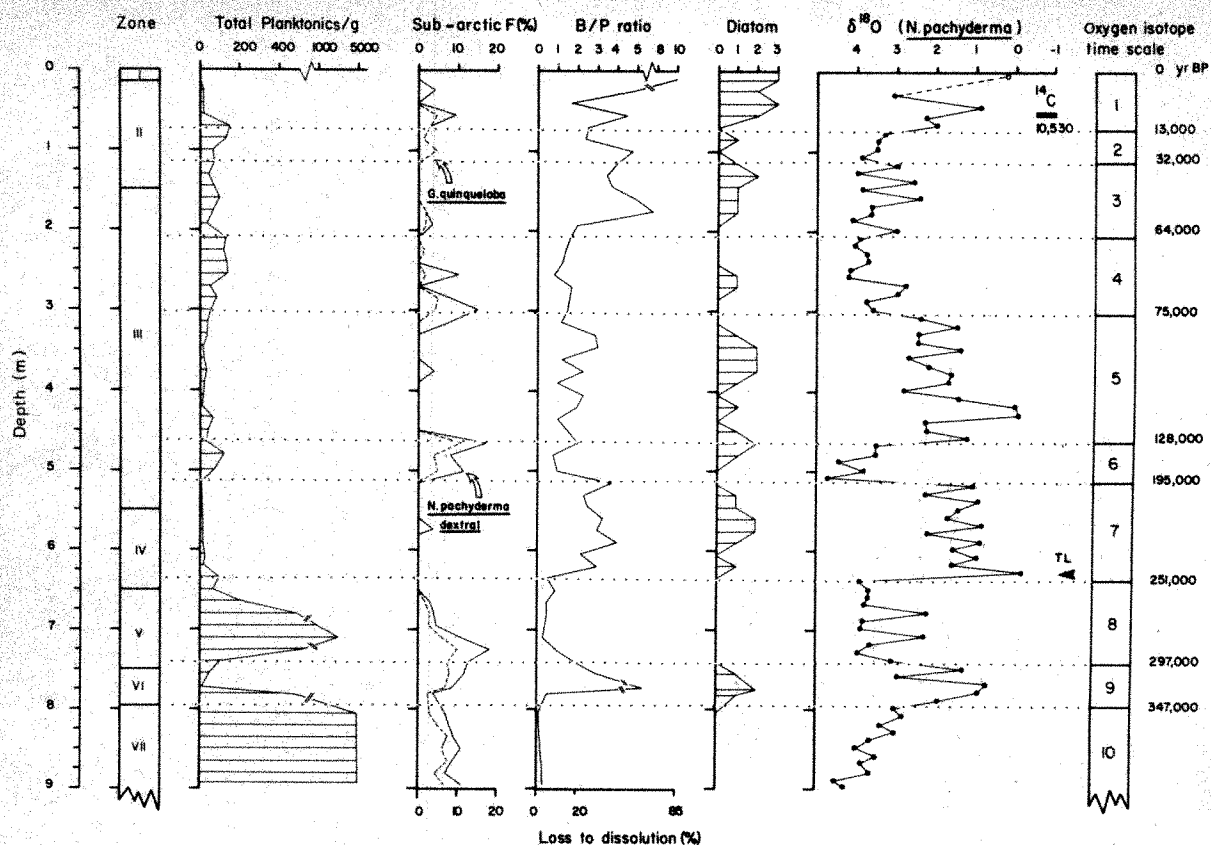


Fig. 2 Foraminiferal zones, planktonic foraminiferal totals per gram sediment, subarctic foraminiferal species (percentage total planktonics), large diatoms (>63 μm) per gram sediment on a scale from 0 (absent) to 3 (abundant, >10,000), and $\delta^{18}\text{O}$ record of *Neogloboquadrina pachyderma* (sinistral) in core 017. Oxygen isotope time scale refers to ref. 11. ^{14}C , radiocarbon data; TL, thermoluminescence data; B/P ratio is the ratio of total benthic to total planktonic foraminifera. Dashed line at top of the isotope record was obtained from the grab sample shown in Fig. 1.

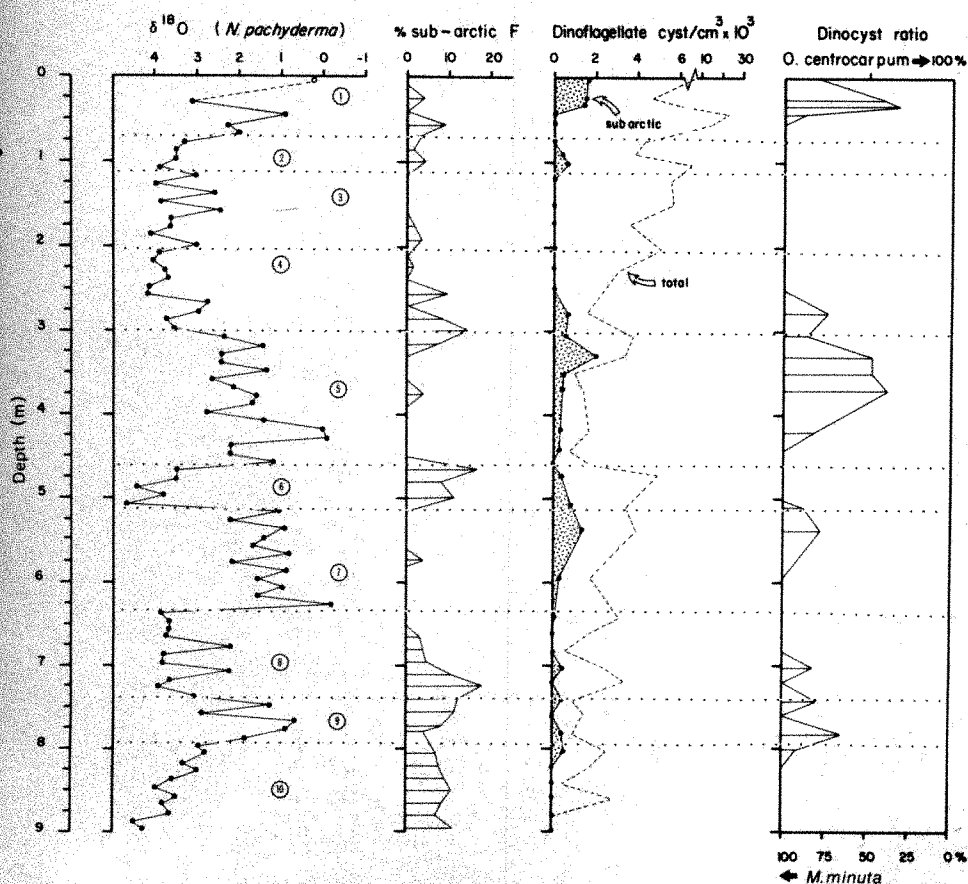


Fig. 3 Summary of dinoflagellate cysts in core 017, compared with the $\delta^{18}\text{O}$ record and percentage total subarctic planktonic foraminifera. See text for explanation of subarctic (=Atlantic) dinoflagellates and the Dinocyst ratio. Circled numbers refer to isotopic stages.

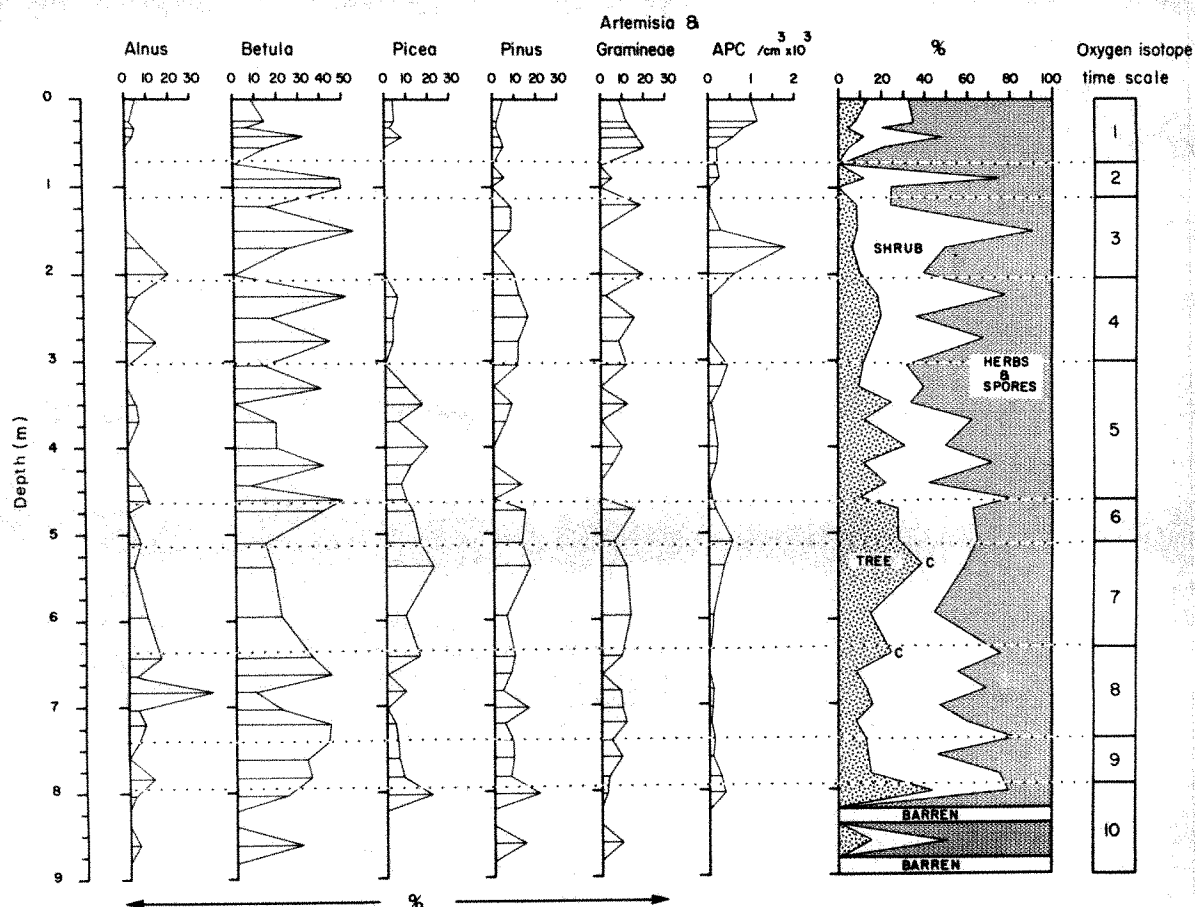


Fig. 4 Pollen percentage diagram for main tree, shrub and herb taxa in core 017, total concentrations of Quaternary pollen and spores (APC) and cumulative percentages of conifer tree, 'shrub' (*Alnus* and *Betula*) and herb pollen plus moss spores. C, *Carya* pollen present.

interglacial stages and are correlated with diatom peaks. These features indicate that maximum primary productivity during the late Quaternary is associated with interglacial stages of relatively warm, open summer water in southern Baffin Bay. The productivity peaks may account for the greater dissolution (20%) of planktonic foraminifera during interglacials than during glacial stages (<10% dissolution)⁷. However, the presence of Atlantic dinoflagellates in stages 2 and lower stages 4, 6 and 8 also implies relatively warm surface water at the core site during early parts of glacial stages, providing independent confirmation of the palaeotemperatures suggested by the subarctic foraminifera.

As for the foraminifera, the number and diversity of quaternary pollen and spores in core 017 (Fig. 4) are low, as expected for oceanic sediments north of the treeline in eastern Canada^{8,9}, but again there are cyclical variations in 'absolute' pollen concentrations (APC) and percentage abundance of indicator species which are correlated with oxygen isotope stages. The top of stage 1 (0–50 cm) is characterized by boreal-subarctic shrub (*Alnus*) and tree pollen percentages (*Pinus*, *Picea*) similar to those found in surface sediments of Baffin Bay⁹, south of the summer position of the Atlantic–Arctic air front (Fig. 1). About 75% of the *Betula* pollen are the small *B. minor* type typical of tundra shrubs. Gramineae dominate the herb pollen, with other taxa including *Artemisia*, *Oxyria*, Rosaceae and Saxifragaceae. The interval from 50 to 325 cm is marked by rare *Picea* and by large fluctuations in *Betula*. Stage 2 contains low APC and variable percentage *Pinus*, Gramineae, *Artemisia*, *Sphagnum* and *Polytrichum*, as presently found in marine sediments⁹ north of the Atlantic–Arctic air front. *Betula* percentages are similar to those found in the Devon Island ice core for the same time interval¹⁴. Stage 3 is marked by higher APC and *Alnus* occurs at the base, suggesting some influx of southern air¹⁵. Stages 4,

8 and 10 contain very low APC but *Alnus* and *Picea* suggest sporadic influx of Atlantic air. Stage 5 is marked by higher tree pollen percentages and peaks in *Picea* and *Alnus*; the assemblages are similar to those in stage 1, but higher percentage *Betula* suggests a wider shrub distribution than presently found on Baffin Island. This agrees with pollen in peat below the Kogalu marine sediments¹⁶, with an age of ~130,000 yr BP.

Tree pollen in stage 6 suggest a strong influx of Atlantic air, but sampling control is poor for this interval in core 017, and data from other cores (Fig. 1) show that this is true only for sediments near the stage 6–7 transition. Interglacial stages 7 and 9 show slight increases in APC and percentage tree pollen; the presence of *Carya* also marks stage 7 in core 017 and other Davis Strait cores¹³.

Overall, the combined palynological and foraminiferal data from core 017 provide palaeoclimatic evidence which supports models^{1–3} that attribute North Atlantic continental ice sheet growth to increased northward flow of warm water during early glacial stages. The present relatively warm climate and sea-surface temperatures in southern Baffin Bay result from (1) high latitude penetration of warm cyclonic air fronts; (2) inflow of Atlantic water through the West Greenland Current. Meridional air flow accounts for boreal tree and shrub pollen in modern Baffin Bay sediments^{9,15} and during older interglacial stages. Lower pollen concentrations and diversity during glacial stages suggest a southward shift of the Arctic air front and stronger zonal flow of polar air characterized by *Betula*, *Artemisia*, Gramineae and other herbs, in addition to ubiquitous *Pinus* grains. However, the presence of boreal tree/shrub taxa in glacial stages 4, 6, parts of 8 and 10 indicate influx of Atlantic air during all initial and some later phases of ice-sheet growth. Presence of Atlantic dinoflagellates during early glacial stages also

supports the foraminiferal data indicating increased water mass transport from the south. Furthermore, the persistent presence of pollen and dinoflagellates during glacial maxima implies extensive areas of summer open water (for example, more than the present ice covered Arctic Ocean north of Ellesmere Island¹⁷), although production of dinoflagellates and large (>63 µm) diatoms is low. Heterotrophic peridinioid dinoflagellates like *M. minuta* (= *Protoperidinium pellucidum* cyst form) can survive under ice up to 2 m thick, but they are very rare or absent in continuously ice covered regions¹⁷.

We conclude that both palynological and foraminiferal studies of core 017 provide palaeoclimatic data which contradict models requiring continuous sea-ice cover in Baffin Bay/Labrador Sea during glacial stages⁵ but are compatible with models based on orbital changes in summer insolation, with very rapid initial glaciation¹⁻³ and subsequent limited sea ice, the continental ice sheets being maintained by precipitation transported northwards from the south-west along storm tracks directed by strong land-sea thermal gradients.

We thank D. Chloe Younger for assistance with core sampling, Larry Ogden for palynological sample preparation, and we acknowledge NSERC support for the QPEC Program at Dalhousie University and GSC support for project 820044. This is Dalhousie University Centre of Marine Geology contribution no. 12.

Received 7 June; accepted 21 September 1984.

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Inheritance of stereotyped gibbon calls

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Little is known about how vocal patterns develop in non-human primates, mainly because suitable controlled experiments are difficult to carry out on these animals¹. Results of isolation experiments²⁻⁴ and observations of interspecific hybrids^{1,5} suggest no greater role for vocal learning than exists in many other vertebrates⁶⁻⁹, and less than has been found in birds¹⁰⁻¹³. We have now studied vocal patterns of hybrids between white-handed gibbons (*Hylobates lar*) and pileated gibbons (*Hylobates pileatus*) in natural mixed-species groups, in a zone of interspecies contact in central Thailand, and in some captive mixed-species groups. We find that in female hybrids, the patterns of the loud and stereotyped 'great-calls' show no evidence of learning from parents, and appear to be under strong genetic control. Daughters maturing in groups with genetically unlike parents develop great-calls unlike those of their mothers, even though these calls develop only while the daughters sing simultaneously with their mothers.

Great-calls are the most spectacular sounds in the repertoires of gibbons. They are sung by females at intervals of 2-4 min

during morning song bouts, and last approximately 14-24 s. In most species, females are accompanied by their mates in a ritualized duet¹⁴⁻²⁰; in a few species, females sing solo near their mates^{16,20-22}. The songs are believed to function in territory advertisement^{5,14,16,23} and in maintaining the monogamous pair bond^{15,19,24-26}.

The acoustic patterns of great-calls are highly species-specific^{16,27,28}. The great-calls of *H. lar* and *H. pileatus* have similar length and frequency range, the major differences being in the shapes and number of notes (Fig. 1). Great-calls of *lar* average 8.4 notes (s.d. 1.66; $n = 14$ individuals recorded in Khao Yai Park, Thailand, one call randomly selected from each) and *pileatus* great-calls average 72.8 notes (s.d. 9.61; $n = 19$ individuals, recorded from three locations in Thailand)²⁹. Comparison of sound spectrograms of *lar*, *pileatus* and hybrid calls shows that the number of notes per s uttered at the end of the call varies relatively less than the total number of notes. For *lar*, the number of notes per s in the last quarter of the call (including no less than three notes) averages 0.49 (s.d. 0.054; $n = 14$) compared with an average of 9.6 (s.d. 0.51; $n = 19$) for *pileatus*²⁹.

Most of our recordings of wild gibbons were made in a small zone of overlap and hybridization between *H. lar* and *H. pileatus* in Khao Yai National Park, central Thailand^{27,29,30}. About 215 groups were identified and mapped in this zone during 1976-80, and 23 groups were found to contain mated adults of different species or hybrids, most of which have been tape recorded. All tape-recorded hybrids have been frequently studied closely with binoculars.

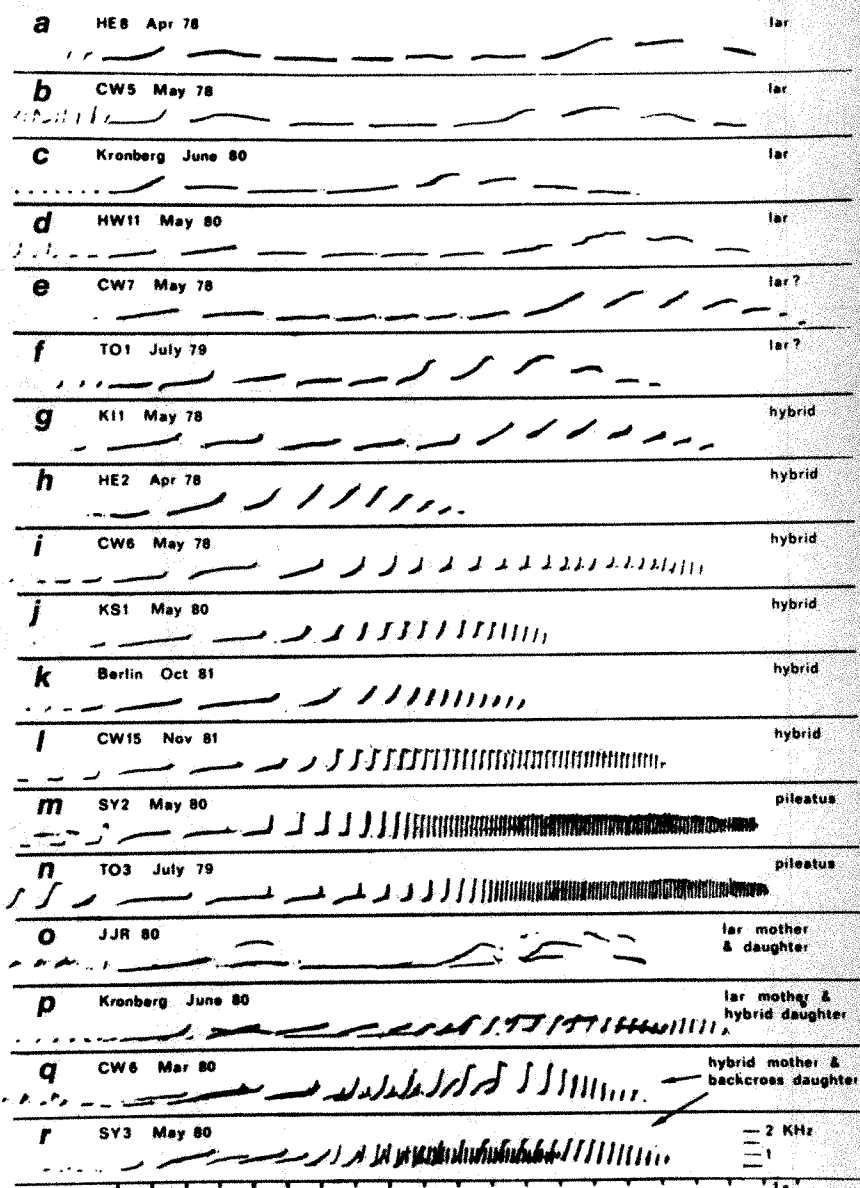
Despite their close relationship, body colour patterns provide unambiguous separation of the species and recognition of their hybrids²⁹. The major pelage differences are the presence of a complete ring of white fur around the face in *lar*, compared with white eyebrows and a black cap in *pileatus*; also a white scrotal tuft of fur in *pileatus* males, and a black 'vest' and greyish body in *pileatus* females, compared with the solid buff or brownish-black body coloration in *lar*. *H. pileatus* is sexually dichromatic whereas *lar* is asexually dichromatic in Khao Yai. Hybrids have various odd or intermediate colour patterns. No individuals with normal *lar* or *pileatus* call types have been found to have odd or atypical coloration, but a few individuals with *lar*-like coloration (for example, females CW7 and TO1, Fig. 1) have slightly more rapid emission of notes than is typical of *lar*.

Great-call patterns of clear phenotypic hybrids tend to be intermediate between those of *lar* and *pileatus* in nearly all respects. Those hybrids that resemble one species more than the other (Fig. 1g, h, i)²⁹ show a corresponding resemblance in great-call pattern. One feature that is somewhat more variable in hybrids than in *lar* or *pileatus* is the length of the call; some have fairly short calls (for example, HE2). In respects other than acoustic pattern, such as frequency and time of singing, duet structure and loudness, the hybrids have not been found to differ significantly from normal *lar* or *pileatus*, although in some respects they are intermediate between the species (our unpublished data).

The relatively rigid territoriality and monogamy of gibbons³¹ allows the identification of mother-daughter pairs in the wild with a high degree of certainty. Juvenile or sub-adult females in the family group frequently sing along with their mother's great-calls, the mother and daughter usually beginning their calls within 1 s of each other^{15,18,22,32-34}. This suggests that the young may have to learn or practise the song³⁵, which does not seem to serve any important function before adulthood.

Three groups with genetically unlike parents (as determined by pelage) had 4-5-year-old daughters who sang with their mothers. The pelage of the daughters was intermediate between that of parental types, providing further evidence of parentage²⁹. Sonagrams of two of these pairs of females are shown (Fig. 1q, r). In all three cases the adult females were hybrids, with call patterns and pelage about half-way between *lar* and *pileatus*. In two cases (CW6 and HS3), the adult male was a *lar*; in the

Fig. 1 Sound spectrograms of representative great-calls of female *H. lar*, *H. pileatus* and hybrids. The great-calls begin near the first 1-s mark on the scale at the bottom; the first 3 s of the sonagrams consist of introductory 'hoos' by the female and (cases *b*, *d*, *o*, *q*) notes by the male partner. When more than one call of a female was available on tape, the one chosen for spectrography was selected randomly. All calls except *c*, *k* and *p* were recorded in Khao Yai Park, Thailand. Great-calls were recorded from four *lar* females (*a*–*d*), two *lar*-like females, possibly backcrosses, with slightly more notes than normal (*e*, *f*), hybrids with odd but somewhat *lar*-like pelage (*g*, *h*), two hybrids with intermediate pelage (*i*, *j*), an F_1 hybrid in the Berlin Zoo (*k*), a wild hybrid with odd but *pileatus*-like pelage (*l*), two *pileatus* females (*m*, *n*), mother and daughter *lar* (*o*), *H. lar* mother and daughter with *pileatus* father (*p*), hybrid mother and backcross daughter with *lar* father (daughter's call is shorter and ends with more horizontal notes) (*q*), hybrid mother and backcross daughter with *pileatus* father (daughter's call is faster) (*r*). Tape recordings in the field were made with a Nagra SNN recorder with Sennheiser MKH 815 microphone; those of the zoo animals were made with a Uher 4000 Report S recorder with M534 microphone. Sound spectrograms were made on a UA-Ubiquitous spectrum analyser (Nicolet Corporation).



third (SY3), he was a *pileatus*. The daughters' calls were a few seconds shorter than the mothers' and not as loud, as is typical of the calls of juveniles.

Group CW6 was first seen and recorded in May 1978, and was again recorded as a trio in March 1980. The daughter's call still differed from her mother's in the slower rate of emission of notes. By 1981, the call had lengthened slightly and become louder, but the pattern remained the same. In the second group with a hybrid adult female and a *lar* adult male, the juvenile female sang a great-call similar to that of the CW6 juvenile. In group SY3, with a *pileatus* adult male, the daughter's great-call was more rapid than that of her mother, and resembled that of female CW15, a *pileatus*-like adult which, judging from pelage, is probably also a backcross to *pileatus*²⁹ (Fig. 1*r*, *l*).

In all of these mixed groups, the hybrid adult females sang vigorously and at least as frequently as normal *lar* and *pileatus* females. The young have therefore heard a daily average of roughly 8–10 great-calls sung by their mothers throughout their lives³¹.

The songs of two zoo hybrids have also been recorded and sonagraphed (Fig. 1*k*, *p*). One is an offspring of a cross between a light-phase *lar* female and a *pileatus* male, in the Kronberg Zoo, West Germany. The hybrid was 10 years old when recorded and was kept with her parents and two brothers in a large

enclosure where the parents and daughter sang regularly as a trio. The daughter had a great-call of somewhat variable length but usually with 20–26 notes, and with a frequency of notes reaching 2.9 s^{-1} , similar in pattern to some recorded in Khao Yai Park. The other hybrid, born in the Zurich Zoo in 1970 but now located in the Berlin Zoo, is the offspring of a dark-phase *lar* female from Thailand and a *pileatus* male. Its great-call usually had around 15–20 notes, and attained a rate of 2.6 notes per s.

The rate of emission of notes in the great-calls of the zoo F_1 hybrids lies approximately half-way between that of the parental species on a logarithmic scale (Fig. 2). Likewise, the wild hybrids with similar call types (CW6, HS3 and SY3 mothers) have backcross daughters with great-calls which regress half-way (or slightly more) to their fathers' species on this scale. Although it is difficult to postulate the unlearned pattern of a hybrid's call, if learning were present we should expect greater similarity to the mother's call in backcrosses either to *lar* or to *pileatus*, or both. No backcross, however, shows greater resemblance to the mother's genetic type. Although the backcrosses available are still few in number, the data are consistent with the hypothesis that genes for great-call pattern have multiplicative effects on the rate of emission of notes and are inherited in a quantitative fashion.

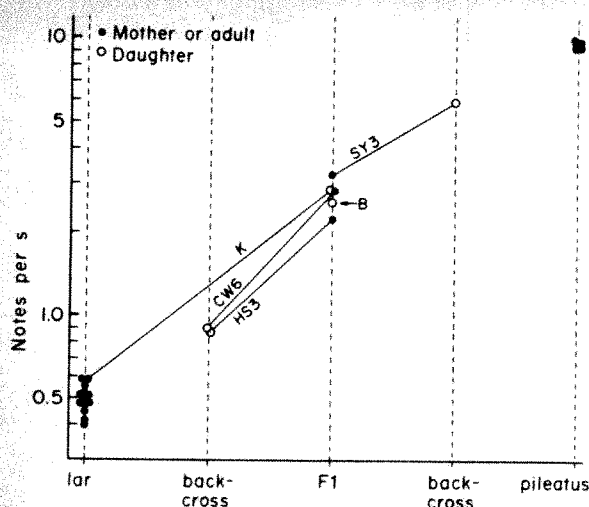


Fig. 2 Number of notes per s in the last quarter of the great-call (including no less than three notes and intervals), on a logarithmic scale in relation to genetic parentage, for *H. lar*, *H. pileatus* and hybrids. The calls of the CW6, HS3 and SY3 adults have been placed as though they were F₁ hybrids, although their parents were not known. The CW6 and HS3 daughters are backcrosses to *lar*; the SY3 daughter is a backcross to *pileatus*. The 12 *lar* and 5 *pileatus* calls were recorded in Khao Yai Park, Thailand, each from a different animal. Mother-daughter pairs are connected by lines. B, Berlin; K, Kronberg.

Intermediate call patterns in hybrids indicates that they could not have strictly learned the patterns from their mothers; had they done so either the *lar* or the *pileatus* call type would have been passed through the generations. As adult males do not give calls resembling great-calls, it is difficult to imagine how the young could have learned great-call patterns from parents at all. The possibility still exists that the young could have been influenced by females in neighbouring groups. This can be ruled out only in the case of the Kronberg female. The female, born in the Zurich Zoo, heard great-calls of her hybrid older sister before she was 2 years old (C. Schmidt, personal communication). Her sister (born in 1961) was also reported to give calls unlike those of her mother, but recordings are not available. No female *pileatus* was in the zoo at this time. As for the wild groups, CW6 was surrounded by *lar* groups, including CW7 (Fig. 1), from 1976–80. The HS3 daughter heard both *lar* and *pileatus* great-calls, and some in the distance that sounded like hybrids. Group SY3 was surrounded by *pileatus* groups, with one or two *lar* groups audible in the distance, but none that sounded exactly like the backcross daughter.

Thus, although singing with parents may be important to the timely development or maturation of the great-call in young females, or for learning its proper timing and context, the acoustic pattern of the final result seems to be resistant to alteration by learning in the natural social setting, and under strong genetic control.

In some respects, great-calls are not typical gibbon vocalizations. No other phrases of the repertoire are as long and stereotyped, although great-calls do display individual variability³⁶. The lack of modification by learning may therefore not be true of all vocal patterns of gibbons. Conceivably, the rigidity of great-call patterns is related to the hypothesized role of duets in strengthening the pair bond, in providing a key stimulus which reinforces the male's fidelity to mate and territory.

We thank the officials of the Berlin (Dr Rahn), Kronberg (Dr Jantschke) and Zurich (Dr C. Schmidt) Zoos for their help, Professor W. Wickler for the opportunity to prepare sonagrams, and R. Ali, C. A. Green and J. J. Raemaekers for comments. Group 'JJR80' was recorded by J. J. Raemaekers. Fieldwork was supported by the New York Zoological Society and the Frankfurt Zoological Society ('Help for Threatened Animals' account).

Received 24 April; accepted 8 October 1984.

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Inositol 1,4,5-trisphosphate microinjection activates sea urchin eggs

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When a sperm activates a sea urchin egg at fertilization, it triggers the release of calcium from a store within the egg by a hitherto unknown mechanism^{1,2}. The ensuing transient increase in cytoplasmic calcium concentration is the stimulus for the beginning of embryonic development^{3,4}. Here we have activated sea urchin (*Lytechinus pictus*) eggs by microinjecting inositol 1,4,5-trisphosphate (InsP₃), a substance which Berridge⁵ has suggested may be an intracellular messenger whose probable function is to release calcium from intracellular stores^{6–9}. It is thought that InsP₃ is produced from plasma membrane phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) via enzymatic hydrolysis by a phospholipase C¹⁰. Recently, it has been demonstrated that there is a substantial increase in the turnover of polyphosphoinositides in the sea urchin egg at fertilization¹¹. We now present preliminary

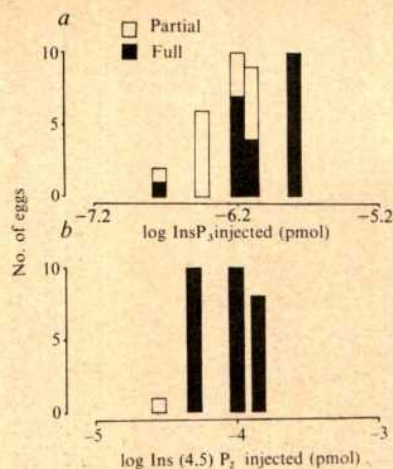


Fig. 1 Elevation of the fertilization envelope induced by microinjection of inositol phosphates: *a*, InsP₃; *b*, Ins(4,5)P₂. The pipette tip was placed eccentrically within the egg. Partial fertilization envelopes arose close to the tip of the pipette. Each amount of InsP₃ indicated was tested by microinjecting 10 eggs; these amounts correspond to 4, 8 or 20 pl of 100 nM, 4 pl of 400 nM or 4 pl of 1,000 nM InsP₃, 8 pl of 20 nM (1.6×10^{-7} pmol) InsP₃ was ineffective. 115 eggs injected with amounts of InsP₃ ranging from 5×10^{-6} to 3×10^{-4} pmol (injected volume 0.5 pl) all showed complete fertilization membranes. 2×10^{-5} pmol Ins(4,5)P₂ was ineffective. The amounts correspond to 1, 4 or 5 pl of 25 μ M or 0.5 pl of 100 μ M Ins(4,5)P₂. Volumes injected were estimated by measuring the diameter of droplets extruded under paraffin oil. Inositol phosphates were prepared as described by Irvine *et al.*²¹; each compound was dissolved in 0.5 M KCl containing 100 μ M EGTA, pH 7.0, and microinjected through bevelled micropipettes using pressure pulses from a nitrogen cylinder⁴. Microinjection of vehicle alone caused activation in 1 of 50 eggs. Free calcium concentration in the injected solutions, measured with a calcium-selective membrane electrode, was $<10^{-7}$ M. Temperature 16 $^{\circ}$ C.

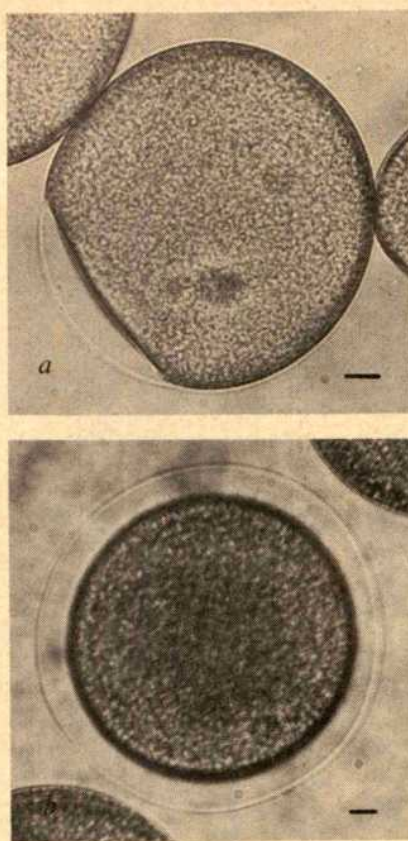


Fig. 2 *a*, A sea urchin egg partially activated by microinjection of 8 pl of 100 nM InsP₃ (8×10^{-7} pmol). Exocytosis has occurred only in a sharply circumscribed region of the egg cortex. *b*, A sea urchin egg wholly activated by microinjection of 0.5 pl of 5 μ M InsP₃ (2.5×10^{-6} pmol). Scale bar, 10 μ m.

evidence that there is a calcium-stimulated breakdown of PtdIns(4,5)P₂ in the egg cortex. We believe that, just as microinjecting InsP₃ stimulates an increase in cytoplasmic calcium, the production of InsP₃ at fertilization may be responsible for the release of calcium from the egg's intracellular store.

The unfertilized sea urchin egg possesses a sensitive *in situ* indicator of cytoplasmic calcium concentration. A cytoplasmic calcium concentration >1 μ M stimulates exocytosis of the egg's cortical granules^{12,13} and a fertilization envelope appears around the egg. Figure 1*a* shows the amount of InsP₃ required to produce fertilization envelopes when injected into unfertilized eggs. Half the activated eggs had complete fertilization envelopes when 20 pl (4% egg volume) of 100 nM or 4 pl (0.8% egg volume) of 400 nM InsP₃ were injected (2×10^{-6} and 1.6×10^{-6} pmol, respectively). Inositol bisphosphates were less effective: Fig. 1*b* shows that 4 and 5 pl of 25 μ M (1 and 1.25×10^{-4} pmol), or 0.5 pl of 100 μ M (0.5×10^{-4} pmol) Ins(4,5)P₂ were needed to cause elevation of the fertilization envelope; 1 pl of 25 μ M Ins(4,5)P₂ (2.5×10^{-5} pmol) caused partial elevation of the fertilization envelope in 1 of 10 eggs (microinjection of vehicle alone caused activation in 1 of 50 eggs) and 1 pl of 10 μ M Ins(4,5)P₂ (10^{-5} pmol) was ineffective. Injection of 40 pl of 25 μ M (10^{-3} pmol) inositol 1,4-bisphosphate (Ins(1,4)P₂) resulted in the partial elevation of a fertilization envelope in only 1 of 16 eggs. These results indicate that InsP₃ stimulates cortical granule exocytosis at concentrations (assuming even distribution) of the order of 10 nM, substantially lower than those reported to release calcium from membrane stores in permeabilized cells, but with similar specificity⁶⁻⁹. InsP₃ seems to stimulate exocytosis indirectly by increasing the cytoplasmic calcium concentration. We determined that InsP₃ has no direct effect by applying it to isolated portions of egg cortex which do

undergo exocytosis when exposed to physiological calcium concentrations¹³.

InsP₃ releases calcium locally: on injection of 4 pl of 400 nM InsP₃ (1.6×10^{-6} pmol), the fertilization envelope in some eggs rose only over the region of the plasma membrane close to the tip of the micropipette. There is a sharp boundary (Fig. 2) between this area of the plasma membrane and surrounding areas where no exocytosis has occurred. This is consistent with a rapid hydrolysis of InsP₃ in the egg cytoplasm, or with a critical threshold concentration for the release of calcium. Injection of smaller volumes of InsP₃ at higher concentration produced the same result: 0.5 pl of 2.5 μ M InsP₃ (1.25×10^{-6} pmol, an equivalent amount) again produced partial elevation of the fertilization envelope.

When a sperm fertilizes a sea urchin egg, the fertilization envelope first appears at the site of sperm entry and rises progressively over the egg surface, appearing at the antipode about 30 s later¹⁴. The asynchronous elevation of the fertilization envelope reflects an underlying wave of cortical granule exocytosis¹⁵. It has been suggested that the increase in cytoplasmic calcium concentration which occurs at fertilization is not spatially uniform, but propagates from the point of sperm entry¹⁶. Figure 3 shows that microinjection of 0.5 pl of 5 μ M InsP₃ (2.5×10^{-6} pmol) induces a wave of exocytosis which begins at the point on the egg surface nearest the tip of the micropipette and reaches the antipode some 25 s later. This observation could be ascribed as readily to diffusion of the injected substance as to the initiation of a propagated wave of calcium release. Figure 3 also shows, however, that the transit time is constant over a fivefold range of InsP₃ concentrations at constant injection volume and is equivalent to the transit time at fertilization in these conditions; it decreases only at a

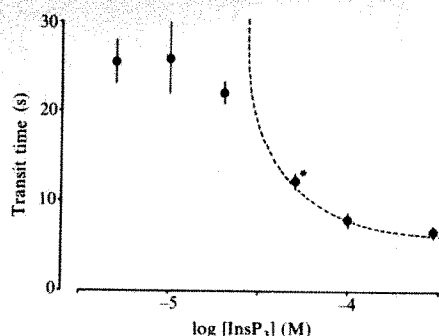


Fig. 3 The dependence of the transit time of the cortical wave of exocytosis on the concentration of InsP_3 microinjected into eggs. The transit time was measured as the time from the first noticeable elevation of the fertilization envelope at the point on the egg cortex nearest the micropipette tip to the time of first noticeable elevation of the fertilization envelope at the point on the egg cortex diametrically opposite the point of initiation. The values are the mean $\pm$ s.e.m. of 20 observations (except for * which is the mean of 15 observations). Volume injected 0.5 pl. Temperature 16 °C. The transit time at fertilization at this temperature in this species is 24 ± 1.5 s (mean $\pm$ s.e.m., $n = 15$). The dotted curve represents the expected relation between transit time and injected concentration if the phenomenon were due to diffusion alone. Two assumptions were made: (1) that the diffusion process can be described by the equations of diffusion from a point source into an infinite medium²²; (2) that a critical InsP_3 concentration must be reached before exocytosis occurs. The first is a reasonable approximation because, although the egg is of finite volume, it is probable that first-order degradation of InsP_3 occurs in the egg cytoplasm. The fit is relatively insensitive to these assumptions, always predicting an initial sharp decline in transit time with increased concentration; it is, however, sensitive to the value of the diffusion coefficient. The dotted curve is drawn for a diffusion coefficient of $3.3 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$, a diffusion path length of 70 μm and a critical concentration of 30 nM. This estimate of the diffusion coefficient agrees well with the expected diffusion coefficient for a molecule the size of InsP_3 in cytoplasm²³.

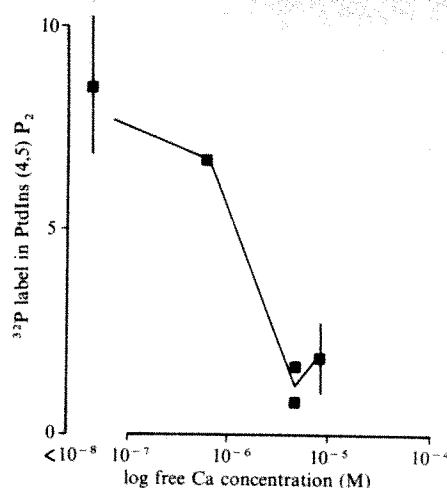


Fig. 4 The calcium dependence of $\text{PtdIns}(4,5)\text{P}_2$ breakdown in cortical fragments isolated from unfertilized sea urchin eggs. A preparation consisting of plasma membrane with associated cortical secretory granules was isolated by attaching eggs to glass coverslips with polylysine and shearing away the bulk of the egg with EGTA-containing medium (220 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 5 mM MgCl_2 , 2.5 mM ATP, 1 mM EGTA, pH 6.7)¹². This preparation allows direct treatment of the cytoplasmic face of the plasma membrane with experimental solutions. After treatment for 2 min with the above medium containing various amounts of free calcium buffered with EGTA¹², the phospholipids were extracted and separated by chromatography²⁴. Chromatograms were developed on oxalate-treated silica gel 60 TLC plates (Merck, Darmstadt) in chloroform/methanol/acetone/acetic acid/water (40:13:15:12:8)²⁵. Lipids were identified by comparison with standards provided by Dr S. Cockcroft. Eggs incubated for 2–3 h in artificial seawater¹² in the presence of ^{32}P -orthophosphate ($200 \mu\text{Ci ml}^{-1}$, 50% v/v suspension; Amersham) incorporated lipid-associated ^{32}P predominantly into $\text{PtdIns}(4,5)\text{P}_2$ and phosphatidylinositol-4-phosphate ($\text{PtdIns}(4)\text{P}$). The radioactive spots corresponding to $\text{PtdIns}(4,5)\text{P}_2$ were identified by autoradiography, scraped from the plate and measured by liquid scintillation counting. The amount of $\text{PtdIns}(4,5)\text{P}_2$ associated with the cortical fragments is expressed as a percentage of the radioactive $\text{PtdIns}(4,5)\text{P}_2$ present in 2 μl of packed eggs. By labelling egg surface proteins with fluorescein isothiocyanate, we have determined that the amount of egg cortex used for each experimental point corresponds to the cortex of 0.14 μl of packed eggs. It seems, therefore, that little breakdown of $\text{PtdIns}(4,5)\text{P}_2$ occurs when the preparation is made in the presence of excess EGTA. The points without error bars represent single determinations. $\text{PtdIns}(4,5)\text{P}_2$ associated with the cortex in low calcium medium ($<10^{-8}$ M calcium) is $8.6 \pm 2.09\%$ (as above) (mean $\pm$ s.e.m., $n = 9$); the amount associated with the cortex 2 min after treatment with 8 μM calcium is $1.9 \pm 0.81\%$ (as above) (mean $\pm$ s.e.m., $n = 8$). This difference is significant ($P < 0.01$, Student's two-tailed t -test). Temperature 16 °C.

concentration between 20 and 50 μM . The volume of solution injected represents 1/1,000 of the egg volume. These observations are consistent with the notion that InsP_3 induces a propagated wave of calcium release similar to that which may occur at fertilization.

One consequence of the increase in cytoplasmic calcium concentration which occurs at fertilization is an increase in cytoplasmic pH¹⁷. The cytoplasmic alkalinization is the second of the two ionic signals which cause activation of the egg², and may be detected by measuring the fluorescence of fluorescein trapped in the egg^{11,18}. Microinjection of 0.5 pl of 10 μM InsP_3 (5×10^{-6} pmol) into unfertilized eggs caused a $116 \pm 2.6\%$ (mean $\pm$ s.e.m., $n = 4$) increase in fluorescein fluorescence, similar to the increase of $119 \pm 3.1\%$ (mean $\pm$ s.e.m., $n = 7$) which occurs at fertilization². Thus, we conclude that InsP_3 sets in motion the ionic changes which constitute egg activation.

Our data demonstrate the physiological effects of InsP_3 in an intact cell. In addition, our experiments supply possible answers to the question of how a sperm activates a sea urchin egg. There is a substantial turnover of $\text{PtdIns}(4,5)\text{P}_2$ at fertilization¹¹. Figure 4 shows that the breakdown of $\text{PtdIns}(4,5)\text{P}_2$ in an egg plasma membrane preparation is stimulated by calcium concentrations of 3–5 μM , the concentration range at which exocytosis occurs in this preparation¹³ and also the calcium concentration measured in the egg cytoplasm at fertilization⁴. Our results are consistent with a mechanism in which InsP_3 -stimulated calcium release and calcium-stimulated InsP_3 production cooperate to produce a wave of calcium release which propagates through the egg cytoplasm.

What sets this process in motion? Dale and co-workers have reported that sea urchin eggs can be activated by microinjecting

the soluble fraction of a sperm homogenate¹⁹. Eggs can be activated by microinjecting micromolar calcium²⁰ but the soluble sperm fraction is reportedly calcium-free. It is an attractive possibility that the sperm introduces InsP_3 or a phospholipase C activity on fusion with the egg, triggering the autocatalytic cycle of calcium release and $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis.

We thank the SERC and the Wellcome Trust for support. We also thank Michael Berridge and Shamshad Cockcroft for the interest they have shown in this work, and Andrew Letcher for his help in the preparation of inositol phosphates.

Received 6 July; accepted 8 October 1984.

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Epithelial cells expressing aberrant MHC class II determinants can present antigen to cloned human T cells

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The first step in the induction of immune responses, whether humoral or cell mediated, requires the interaction between antigen-presenting cells and T lymphocytes restricted at the major histocompatibility complex (MHC)^{1,2}. These cells invariably express MHC class II molecules (HLA-D region in man and Ia in mouse) which are recognized by T cells of the helper/inducer subset in association with antigen fragments^{3,4}. Interestingly, in certain pathological conditions⁵⁻⁸, for example in autoimmune diseases such as thyroiditis⁵ and diabetic insulinitis⁶, class II molecules may be expressed on epithelial cells that normally do not express them. We speculated that these cells may be able to present their surface autoantigens to T cells, and that this process may be crucial to the induction and maintenance of autoimmunity⁹. A critical test of this hypothesis would be to determine whether epithelial cells bearing MHC class II molecules (class II⁺ cells) can present antigen to T cells. We report here that class II⁺ thyroid follicular epithelial cells (thyrocytes) can indeed present viral peptide antigens to cloned human T cells.

We have described recently a T-cell clone (HA1.7) that is specific for a defined peptide (p20; residues 306-329) of the HA-1 molecule of influenza strain A haemagglutinin (subtype H₃N₂)¹⁰. In the presence of antigen-presenting cells, these T cells proliferate in response to the intact influenza virus of the appropriate subtype. As the antigenic determinant recognized by HA1.7 (p20) is also present in the intact virus, these cloned T cells provide a sensitive and homogeneous assay for the capacity of other cell types to function as antigen-presenting cells. However, the induction of T-cell proliferation to either the intact virus or p20 requires recognition of the relevant MHC class II polymorphism (for HA1.7 this is an HLA-DC/DS (now termed HLA-DQ) specificity in linkage disequilibrium with HLA-DR1; ref. 11). It was essential, therefore, to select potential antigen-presenting cell populations from a histocompatible donor.

We used epithelial thyroid cells from a patient with Graves' disease (hyperthyroidism), because this disorder is commonly treated by surgery, and thyroidectomy glands are a good source of MHC class II⁺ thyrocytes. Peripheral blood mononuclear leukocytes (PBL) from the same thyroid donor (with the

appropriate HLA-DR phenotype) were assayed for their ability to present antigen to clone HA1.7. Irradiated PBL induced substantial proliferative responses of HA1.7 to either influenza A/Texas virus or p20 (Fig. 1). Viable thyrocytes from the same donor, after the removal of non-adherent cells, were stained and found to be positive for MHC class II molecules. They were identified as thyroid epithelial cells by staining with Hashimoto's serum, known to contain antibodies to the microvillar/microsomal antigens expressed on thyrocytes¹². No contaminant macrophages were detected using monoclonal antibodies, thus excluding the possibility that they were acting as antigen-presenting cells. The percentage of DR⁺ thyrocytes could not be determined precisely, because cells were in confluent monolayers, but fluorescence microscopy showed it to be in the range of 60-80%.

When these thyrocytes were tested for their capacity to present intact influenza virus and p20 to T cells of clone HA1.7, there was a reproducible marked proliferative response to p20. This effect was also tested over a limited dose range of thyrocytes (10³ or 10⁴) (Fig. 1), because in certain circumstances the number of antigen-presenting cells is critical¹³. Unlike the results obtained with the PBL from the thyroid donor (Fig. 1), there was no response to whole formalin-inactivated A/Texas influenza virus (Fig. 2) over a wide dose range.

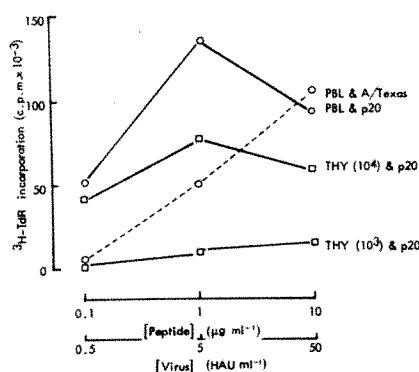


Fig. 1 Presentation of intact influenza virus and peptide to cloned T cells by histocompatible peripheral blood leukocytes from a patient with Graves' disease.

Methods: T-lymphocyte clones specific for synthetic peptides of influenza haemagglutinin (HA) were isolated as previously described. Briefly, PBL were cultured for 6 days with 0.1 $\mu\text{g ml}^{-1}$ HA (gift of Dr R. G. Webster). Following enrichment on a discontinuous Percoll (Pharmacia) gradient, lymphoblasts were resuspended in RPMI 1640 (Gibco) containing 10% A⁺ serum and T-cell growth factor (TCGF) and plated at one cell every third well in Microtest II trays (Falcon) with 10⁴ irradiated autologous PBL and 0.1 $\mu\text{g ml}^{-1}$ HA. After 7 days, growing clones were transferred to 96-well microtitre trays and then to 24-well trays. At each transfer the clones received fresh TCGF and autologous irradiated PBL together with specific antigen. The clones were expanded in 25-cm² tissue culture flasks receiving irradiated PBL and intact virus (A/Texas/1/77) every 7 days and fresh TCGF every 3-4 days. TCGF was prepared from 48-h supernatants of PBL ($1 \times 10^6 \text{ ml}^{-1}$) cultured with 0.1% purified phytohaemagglutinin (Difco) in complete culture medium containing 2.5% A⁺ serum. Before use in proliferation assays, the clones were rested 6-7 days after the addition of irradiated filler cells. For proliferation assays¹⁸, cloned T cells ($5 \times 10^4 \text{ ml}^{-1}$) were cultured with irradiated PBL ($20 \times 10^4 \text{ ml}^{-1}$) in the presence of p20 (0.1-10 $\mu\text{g ml}^{-1}$, ○—○), A/Texas (0.5-50 HAU ml^{-1} , ○---○) or thyrocytes (10⁴ or 10³, □—□) in the presence of p20 0.1-10 $\mu\text{g ml}^{-1}$. Following 60 h incubation, the cultures were pulsed for 8-16 h with 1.0 $\mu\text{Ci ml}^{-1}$ of ³H-thymidine (TdR, Amersham) and collected onto glass fibre filters. Proliferation as correlated with ³H-TdR incorporation was measured by liquid scintillation spectroscopy. Results are expressed as mean counts per min for triplicate cultures. Background response of cloned T cells alone was 271 ± 5 and in the presence of IL-2 it was $25,672 \pm 729$ (arithmetic mean $\pm$ s.d.).

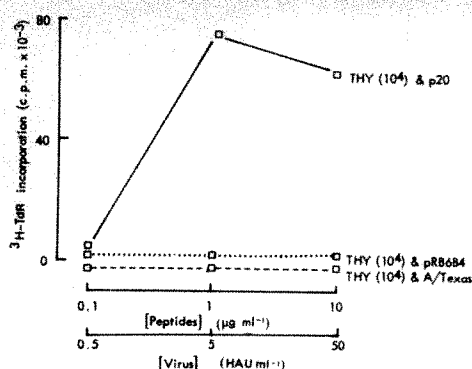


Fig. 2 Response of HA1.7 to inactivated influenza virus and p20 in the presence of histocompatible thyrocytes.

Methods: Irradiated (3,000 rad) thyrocytes from the same donor as for the PBL were prepared by collagenase type IV (Worthington) digestion at 37 °C (5 mg ml⁻¹) for 3 h as described in more detail elsewhere¹⁹, in the absence of any serum. The thyrocytes (10⁴ per well) were plated in 96-well flat bottom microtitre trays and after 1 day, washed to remove any non-adherent cells that may be able to present antigen. They were then cultured with T-lymphocyte clones specific for synthetic peptide of influenza haemagglutinin (HA1.7)¹⁰, in the presence of p20 (0.1–10 μg ml⁻¹, □—□) or formalin-inactivated influenza A/Texas (0.5–50 HAU ml⁻¹, □---□). A non-stimulatory influenza peptide of the HA-1 molecule (RB6B4, residues 314–329) was used as a control (□···□). Proliferation was measured by thymidine incorporation, as described in Fig. 1 legend. The degree of macrophage/monocyte contamination was checked using the monoclonal antibodies TG1 and UCHM1 (ref. 20). The response of HA1.7 alone was 114 ± 77, and in the presence of IL-2 it was 40,813 ± 4,930 (arithmetic mean ± s.d.).

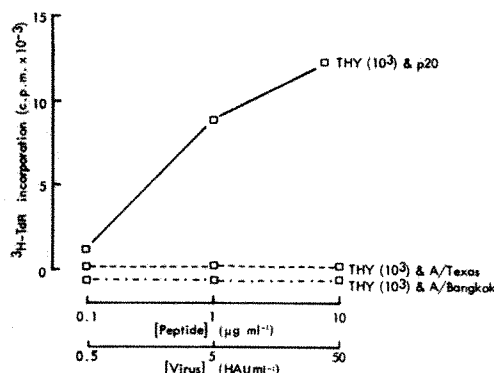


Fig. 3 Response of HA1.7 to infectious inactivated influenza virus and p20 to the presence of histocompatible thyrocytes.

Methods: Cloned T cells (HA1.7) were cultured with infectious influenza A/Bangkok (0.5–50 HAU ml⁻¹, □---□), inactivated influenza A/Texas (0.5–50 HAU ml⁻¹, □--□) or p20 (0.1–10 μg ml⁻¹, □—□) in the presence of irradiated thyrocytes (10³ per well). The background response of HA1.7 alone or in the presence of IL-2 were, respectively, 393 ± 259 and 22,417 ± 7,839.

After 24 hours the thyrocyte cultures were vigorously washed 3 times prior to the addition of T cells and antigen, in order to remove non-adherent cells, confirming that the presenting capacity was due to adherent thyrocytes and not to non-adherent MHC class II⁺ cells such as B cells or activated T cells. Dendritic cells, which are important antigen-presenting cells in some proliferative responses, are adherent at 2 h but not at 18 h, and thus would have been expected to be removed by this procedure^{14,15}. Furthermore, we have found recently that mouse dendritic cells, unlike human thyrocytes, can present particulate antigens such as red cells and mycobacteria¹⁶ and can process proteins such as keyhole limpet haemocyanin (B. Chain, P. Kaye & M.F.,

Table 1 Inhibition of thyrocyte-induced response to p20 by anti-class II antibody

Cells	Antigen	Antibody	Response (c.p.m. ± s.d.)
HA1.7	—	—	114 ± 77
HA1.7	—	IL-2	40,813 ± 4,930
HA1.7	Thyrocytes	RB6B4	1,110 ± 571
HA1.7	Thyrocytes	p20	75,041 ± 14,844
HA1.7	Thyrocytes	p20	3,460 ± 610
HA1.7	—	p20	1,112 ± 90
—	Thyrocytes	—	142 ± 26

Cloned T cells (10⁴ per well) were cultured with MHC class II⁺ irradiated (3,000 rad) thyrocytes (10⁴ per well) in the presence of p20 or RB6B4 (1 μg ml⁻¹). Anti-HLA class II ascites (HIG78)²¹, which is known to block the response of HA1.7 (ref. 11), was included from the start of the cultures, at a dilution of 1/200. Proliferation was determined as described in Fig. 1 legend. Control responses of HA1.7 alone or with added interleukin-2 (IL-2) are shown.

manuscript in preparation). It seemed unlikely that monocyte/macrophage-like cells in the thyrocyte preparations were involved, because these were not detected by staining with monoclonal antibodies.

As thyrocytes failed to present formalin-inactivated A/Texas virus (Figs 2, 3), it was of interest to determine whether they were able to present an infectious preparation of A/Bangkok, which contains p20 of identical sequence to that of influenza A/Texas. The thyrocytes did not present infectious virus (Fig. 3), but did present p20 to HA1.7. The inability of MHC class II⁺ thyrocyte preparations to present either infectious or inactivated influenza virus, in contrast to PBL, indicates that the antigen presentation noted could not have been due to any undetected contaminating MHC class II⁺ PBL. Although it was conceivable that the failure of thyrocytes to present influenza A virus was due to their inability to bind and take up influenza virus, this seemed unlikely, as influenza virus, which uses neuraminic acid as cellular receptor, can infect virtually all cell types¹⁷. This possibility was excluded by the strong staining of influenza A virus-incubated thyrocytes with rabbit anti-influenza A/Bangkok antiserum.

To verify that the presentation of p20 by thyrocytes was an immunologically specific reaction and not a mitogenic effect perhaps due to thyroid hormones, another peptide (RB6B4) from the same virus sequence but which does not activate HA1.7, was used as antigen, without effect (Fig. 2). To confirm that the expression of MHC class II antigen by thyrocytes was essential to their presenting function, an anti-class II monoclonal antibody, which inhibits the response of HA1.7 to PBL used as antigen-presenting cells¹¹, was used in co-culture experiments. It efficiently inhibited the response of HA1.7 to p20 which was induced by thyrocytes acting as antigen-presenting cells (Table 1).

The capacity of thyrocytes to present peptide antigen clearly indicates the functional nature of the MHC class II molecules detected on the surface of thyrocytes by immunofluorescence with monoclonal antibodies, and thus we can anticipate that other cells expressing aberrant HLA-DR or Ia molecules in other disease states^{6–9} may in the appropriate circumstances act as autoantigen-presenting cells. The restriction on the type of antigen presented by DR⁺ thyroid epithelial cells is noteworthy. These cells were capable of presenting only peptides which did not need further catabolism or 'processing' in order to expose their antigenic determinants, but could not present intact virus, which requires processing. This suggests that, *in vivo*, class II⁺ epithelial cells may be able to present all antigens, but only those that do not need further alteration. Thus, surface autoantigens already expressed in the plasma membrane could be efficiently presented to potentially autoreactive T cells. Indeed, we have demonstrated recently that autoreactive T cells, cloned from the intra-thyroid lymphocytes of autoimmune thyroiditis, are re-stimulated by their autologous MHC class II⁺ thyrocytes,

but not by histoincompatible thyrocytes (M.L., G.F.B. and M.F., manuscript submitted).

The restricted antigen-presenting function of class II⁺ epithelial cells is compatible with the need for specialized antigen-presenting cells, such as macrophages¹ and dendritic cells¹⁴ which can process^{3,16} antigen, in generating immune responses to foreign antigens such as viruses. These experiments strongly support the concept that aberrant MHC class II expression is important in perpetuating, and perhaps initiating autoimmune reactions⁹, and indicates that control of this expression may be of importance in abrogating autoimmunity.

We thank Mr Russell for access to his patients and surgical specimens, Josefina Weingast Johnson for assistance, Drs P. C. L. Beverley, J. Bodmer, W. Bodmer, M. Crumpton and V. Van Heyningen for gifts of monoclonal antibodies, Drs R. A. Lerner, N. Green, R. G. Webster, J. J. Skehel and A. S. Rosenthal for providing viral antigen and peptides, and Dr M. Contreras and her colleagues at the National Blood Transfusion Centre, Edgware for their generous assistance. This work was supported by the Imperial Cancer Research Fund, Juvenile Diabetes Foundation (USA), British Diabetic Association, Novo (Denmark), NIH (USA) and MRC. M.L. is supported by the Juvenile Diabetes Foundation.

Received 5 July; accepted 8 October 1984.

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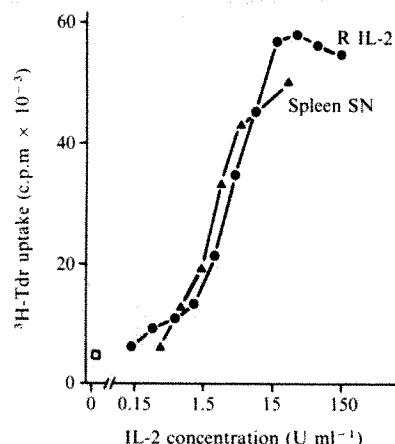


Fig. 1 B-cell growth factor activity of human IL-2. BCGF activity was measured using the assay system described by Muraguchi and Fauci⁹, with minor modifications¹⁰. This system is based on culturing B cells for 72 h with formaldehyde-killed *S. aureus* Cowan strain I before addition of tested growth factors. The *S. aureus* was grown in trypticase soy broth, inactivated by formaldehyde and used at a final dilution of 1:4,000. B-cell populations were represented by non-adherent spleen lymphocytes that had been depleted twice of cells forming rosettes with neuraminidase-treated sheep erythrocytes as described elsewhere¹⁶. The resulting cells contained <1% erythrocyte-rosetting cells. Further analysis of this population was performed using FACS (see Fig. 2). These B cells (5×10^4) were cultured in round-bottomed microtitre trays in 0.2 ml RPMI 1640 with 10% fetal calf serum (FCS) in the presence or absence of *S. aureus*. Serial dilutions of standard spleen supernatant (Δ — Δ) or recombinant IL-2 ($\bullet$ — $\bullet$) were added at day 3. Cultures were continued for an additional 72 h and 0.5 μ Ci of tritiated thymidine (^{3} H-TdR) was added 18 h before collection and measurement of thymidine incorporation. As an indicator cell system for the determination of IL-2 biological (TCGF) activity, we used the CTLL murine cytolytic T lymphocyte line known to proliferate in response to human IL-2 (but not to PHA)¹. These cells were cultured for 24 h in Dulbecco's modified Eagle's medium and 5% FCS in the presence of serial dilutions of tested supernatant. IL-2 activity was assessed by ^{3} H-TdR uptake after 24 h, following a 6-h pulse with 0.5 μ Ci ^{3} H-TdR. The value of 1 U ml⁻¹ was assigned to an IL-2 concentration leading to 50% maximal ^{3} H-TdR uptake. The recombinant human IL-2, purified and free of endotoxin (specific activity 1 U per 0.3 ng protein), was provided by Biogen. The cloning and screening of human IL-2 cDNA plasmid obtained from RNA isolated from PHA-stimulated human spleen cells and its expression in *E. coli* have been performed by Devos *et al.*⁸.

Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells

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Human interleukin-2 (IL-2) is a glycoprotein of relative molecular mass (M_r) 15,000, which is released by T lymphocytes on stimulation with antigen or mitogen and functions as a T-cell growth factor (TCGF) by inducing proliferation of activated T cells¹. It is generally accepted that resting or activated B cells do not respond directly to IL-2 but require for their proliferation other T-cell-derived lymphokines usually referred to as B-cell growth factors (BCGFs)^{2,3}. Recently, however, a monoclonal antibody reacting

with the IL-2 receptor molecules expressed by activated T cells (anti-Tac)^{4,5} was shown to react also with certain B tumour cells⁶; in addition, murine B cells proliferate in response to pure human IL-2⁷. We now show that recombinant IL-2, derived from *Escherichia coli* expressing the human gene⁸, is able to promote strong proliferation of human B cells activated with protein-A-rich *Staphylococcus aureus* Cowans strain I². Moreover, we demonstrate that the anti-Tac antibody also reacts with *S. aureus*-activated normal B cells and inhibits sharply the proliferative response of such cells to IL-2. Finally, immunoprecipitation experiments reveal that anti-Tac defines similar molecules on activated T and B cells.

BCGF activity is measured by an assay system described previously⁹, with minor modifications. This system is based on the growth factor-induced proliferative response of B cells pre-activated for 3 days with an appropriate source of formaldehyde-killed protein A-rich *S. aureus* Cowans strain I. Cell proliferation is evaluated by ^{3} H-thymidine incorporation after an additional 3-days' culture. The ability of recombinant IL-2 to promote T- or B-cell proliferation is compared with that of standard phytohaemagglutinin (PHA)-induced spleen supernatant, routinely used in our laboratory as a source of both TCGFs and BCGFs¹⁰. First, we determined the TCGF activity of the samples examined by a proliferation assay using the CTLL murine cytolytic T-lymphocyte line, known to proliferate in response

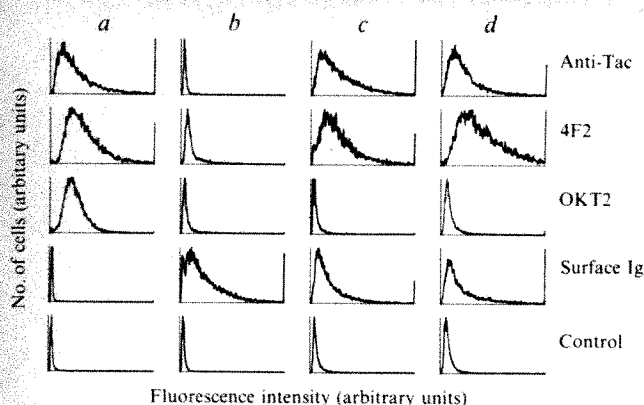


Fig. 2 Anti-Tac antibody binds to activated B cells. Human spleen B cells, isolated as described in Fig. 1 legend, were analysed for surface antigen expression either directly (b), or after 3 days of activation with *S. aureus* (c) or after an additional 3 days of culture in the presence of 15 U ml^{-1} recombinant IL-2 (d) (see Fig. 1). T cells that had been stimulated for 3 days with 1% PHA and then cultured for a further 3 days with recombinant IL-2 (15 U ml^{-1}) were analysed for comparison (a).

Methods: 2×10^5 cells were incubated with one of the following monoclonal antibodies: anti-Tac (100 μl of 1:10,000 ascites in medium), 4F2 (ref. 11) (100 μl of 1:2,000 ascites in medium), OKT3 (5 μl of the standard solution). Control cells were incubated in culture medium with no antibody. Cells were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin for 30 min¹⁵. To detect surface immunoglobulin, cell samples were incubated with the F(ab')₂ of a goat anti-human immunoglobulin¹⁰. Samples were then washed and analysed on a BD FACS system as described previously^{15,17}. Note that both activated B-cell populations expressed the Tac (or the 4F2) antigen to a level similar to that of activated T cells; in contrast, no Tac (or 4F2) antigen was detectable in resting B cells. The B-cell nature of the *S. aureus*-activated cell population was indicated by the absence of T3⁺ cells by the expression of surface immunoglobulin.

to human IL-2¹. Serial dilutions of these samples were then tested for BCGF activity in the *S. aureus*-activated B-cell assay. B-cell proliferation was promoted by both sources of IL-2 (Fig. 1) and we found a strong correlation between BCGF and TCGF activities of the two samples. We examined whether proliferation of contaminating T lymphocytes could account for the response to IL-2 observed in the activated B-cell assay. T lymphocytes were counted as cells forming rosettes with sheep erythrocytes and/or as T3-positive cells by analysis using fluorescein-activated cell sorting (FACS). There were always <1% erythrocyte-rosetting lymphocytes in the cell populations tested before culture and <2% at the end of culture (six different experiments); in addition, T3⁺ cells could not be detected by flow microfluorometry (see also Fig. 3). Thus, we conclude that the ³H-thymidine incorporation observed in *S. aureus*-activated B-cell populations after exposure to IL-2 reflects proliferation of B cells and not contaminating T cells.

Given the ability of recombinant IL-2 to induce proliferation of activated B cells, it was of interest to determine whether binding of IL-2 to B cells occurred via surface receptors similar to those expressed by activated T cells. B cells activated by *S. aureus* were therefore analysed for their reactivity with the anti-Tac monoclonal antibody, which is specific for the IL-2 receptors expressed by activated T cells^{4,5}. Previous studies indicated that other surface molecules expressed by activated T cells, such as the 120,000-*M_r* 4F2, were also expressed by B cells following activation with *S. aureus*¹¹. The expression of Tac and 4F2 antigens was assessed by indirect immunofluorescence and flow microfluorometry on B cells either 3 days after activation with *S. aureus* or after an additional 3-days' culture in the presence of recombinant IL-2. Fresh unstimulated B cells and PHA-activated T cells were analysed for comparison. Both

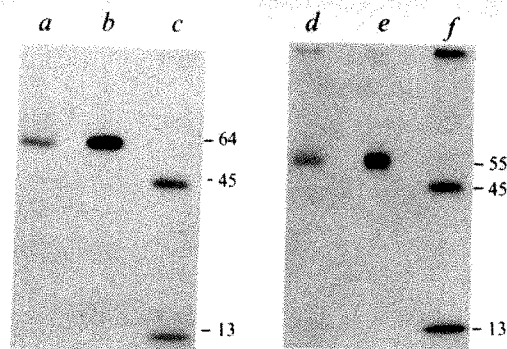


Fig. 3 Anti-Tac antibody recognizes similar molecules on activated T or B cells. a, b, Electrophoretic patterns obtained following immunoprecipitation, in reducing conditions, of ¹²⁵I-labelled B and T cells, respectively, with anti-Tac monoclonal antibody. d, e, The same molecules immunoprecipitated in non-reducing conditions. Migration of HLA class I molecules immunoprecipitated from *S. aureus*-activated B cells with the B9.12 antibody in reducing (c) or non-reducing (f) conditions is shown for comparison. Note that the apparent *M_r* of Tac antigen in reducing conditions is ~64,000 in both cell types, while the *M_r* in non-reducing conditions is 55,000, thus providing evidence for the existence of internal disulphide bond(s) in both T and B cells.

Methods: Spleen B cells were cultured with *S. aureus* for 3 days (see Fig. 1) and with recombinant IL-2 (15 U ml^{-1}) for an additional 48 h. Cells from several microwell plates were pooled and surface labelled with ¹²⁵I using lactoperoxidase-glucose-oxidase-catalysed iodination¹². T cells cultured for 3 days with 1% PHA and for an additional 3 days with recombinant IL-2 were also surface-labelled. Approximately 20×10^6 cells were washed and resuspended in 1 ml phosphate-buffered saline (PBS) containing glucose (5.5 mM) and the following reagents: 20 μl KI ($5 \times 10^{-5} \text{ M}$), Na¹²⁵I (1 mCi), 40 μl lactoperoxidase (1 mg ml⁻¹ solution in PBS), 10 μl glucose oxidase (stock solution diluted 1:100 in PBS). Cells were incubated at 4°C. After 10 min and 20 min, 10 μl glucose oxidase were added and the incubation was carried out for a total period of 30 min. Cells were then washed five times in cold RPMI, twice in PBS and lysed in 10 mM Tris-buffered saline (pH 7.5) containing 1% NP40, 1 mg ml⁻¹ bovine serum albumin and 0.1 mM phenylmethylsulphonyl fluoride (PMSF) for 20 min at 4°C. The supernatant of a 30-min spin at 100,000g was then immunoprecipitated as follows. Supernatants were dialysed with PBS containing 0.05% NP40 and 0.1 mM PMSF and precleared twice with 100 μl of packed protein-A-Sepharose beads for 2 h under rotation. Aliquots (200 μl) were then incubated for 2 h with 10 μl of a 1:10 dilution of anti-Tac or B9.12 (anti-HLA)¹⁸ ascites. 20 μl of protein-A-Sepharose beads were added and samples incubated for 2 h at 4°C. The immunoprecipitate was eluted from protein-A-Sepharose by boiling for 2 min in 5% SDS in the presence or absence of 5% 9-mercaptoethanol and analysed on 11% discontinuous SDS-polyacrylamide gels¹⁹.

activated B-cell populations expressed the 4F2 and Tac antigens to a similar level to that observed on activated T cells (Fig. 2). On the other hand, neither antigen was detected on unstimulated B cells. That the cells expressing Tac antigen in the *S. aureus*-activated cell populations were indeed B cells (and not T cells) was confirmed by the demonstration that most cells expressed surface immunoglobulins whereas T3⁺ cells were virtually absent.

In view of the strong reactivity of anti-Tac with activated T and B cells, we then compared the anti-Tac-reactive molecules present in the two lymphocyte populations. B cells stimulated with *S. aureus* and PHA-induced T-cell blasts were surface labelled with ¹²⁵I by the lactoperoxidase-glucose oxidase-catalysed iodination method¹². They were then lysed and the resulting cell extracts immunoprecipitated with anti-Tac. On SDS polyacrylamide gel electrophoresis (PAGE), the molecules recognized by anti-Tac in both lysates displayed a *M_r* of ~55,000 in nonreducing conditions (Fig. 3, lanes a, b) and 64,000 in reducing conditions (lanes d, e). We therefore conclude that the molecules serving as IL-2 receptors on activated T and B cells are similar.

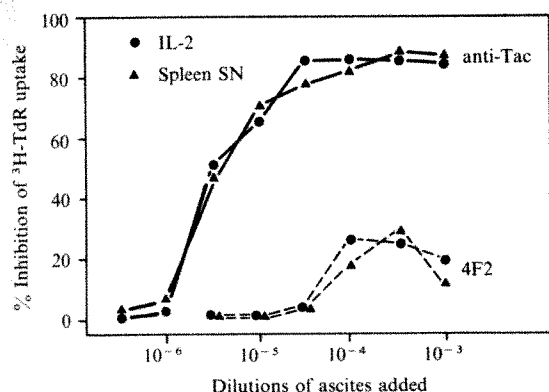


Fig. 4 Anti-Tac inhibits the IL-2-induced proliferation of *S. aureus*-activated B cells. Serial dilutions of anti-Tac or anti-4F2 were added to 5×10^4 spleen B cells that had been cultured for 3 days with optimal concentrations of *S. aureus* in round-bottomed 96-well microtitre plates. Spleen supernatant or recombinant IL-2 were added subsequently at a final concentration of 7 U ml^{-1} IL-2. Triplicate cultures were incubated for 72 h at 37°C and 0.5 mCi of ^3H -TdR was added 18 h before collection (see also Fig. 1). Data are expressed as % inhibition of the response obtained in cultures receiving spleen supernatant or recombinant IL-2 alone ($53,500 \pm 3,400 \text{ c.p.m.}$ and $46,800 \pm 1,400 \text{ c.p.m.}$, respectively).

We next investigated whether the IL-2-induced B-cell proliferative response could be inhibited by anti-Tac (in the absence of complement). The B-cell proliferative response to recombinant IL-2 or spleen supernatant was strongly inhibited by the addition of anti-Tac at a final dilution of 10^{-3} of anti-Tac ascitic fluid whereas anti-4F2 ascites, used as control, had minimal inhibitory effect even when added at final dilutions as high as 10^{-3} (Fig. 4). According to previous studies, supernatant of lectin-stimulated lymphocytes should contain lymphokines with BCGF activity in addition to IL-2. However, in our study, up to 90% inhibition of B-cell proliferation was achieved with the anti-Tac. Taken together, the present results suggest that IL-2 may be responsible for a large part of the BCGF activity generated by PHA-stimulated human spleen cells. Our data do not rule out the possibility that molecules other than IL-2 display BCGF activity. For example, supernatants of lectin-stimulated 72-h lymphocyte cultures have been reported to contain strong BCGF activity but little TCGF activity²; in addition, T-T-cell hybridomas have been described which released BCGF and not IL-2^{13,14}. In any case, limiting dilution analysis of the precursors of IL-2-producing cells has previously indicated that as many as 60% of peripheral blood T cells have this functional potential¹⁵, and it is therefore evident that a large fraction of human T cells can also influence B-cell responses via IL-2.

We thank Dr J. C. Cerottini for helpful discussion, Dr A. S. Fauci for the 4F2 antibody and Dr B. Malissen for the B9-12 (anti-HLA) antibody, J. Hosking for technical help and M. van Overloop for secretarial assistance.

Received 13 August; accepted 8 October 1984.

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Production of functional chimaeric mouse/human antibody

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The availability of monoclonal antibodies has revived interest in immunotherapy. The ability to influence an individual's immune state by administering immunoglobulin of the appropriate specificity may provide a powerful approach to disease control and prevention. Compared with immunoglobulin from other species, human immunoglobulin (Ig) might be best for such therapeutic intervention; it might function better with the recipient's effector cells and should itself be less immunogenic. The success of the mouse hybridoma system suggests that immunoglobulin of virtually any specificity can be obtained from a properly immunized animal. In the human system, however, immunization protocols are restricted by ethical considerations, and it is not yet clear whether human antibody-producing cell lines of the required specificity can be obtained from adventitiously immunized individuals or from *in vitro* immunized cells. A method which might circumvent these difficulties is to produce antibodies consisting of mouse variable regions joined to human constant regions. Therefore, we have constructed immunoglobulin genes in which the DNA segments encoding mouse variable regions specific for the hapten trinitrophenyl (TNP) are joined to segments encoding human μ and κ constant regions. These 'chimaeric' genes are expressed as functional TNP-binding chimaeric IgM. We report here some of the properties of this novel IgM.

The variable regions used in the experiments described here are derived from the hybridoma cell line Sp6 which secretes IgM(κ) specific for TNP¹. The specific κ gene² and μ gene³ have been cloned and were used as a source of TNP-specific variable regions which were joined to cloned human μ and κ constant regions^{4,5} in the vector pSV2-neo⁶ (Fig. 1).

To assay the chimaeric light- and heavy-chain genes independently of each other, we transferred these genes into appropriate mutant hybridoma cell lines derived from Sp6. The vector pN $\cdot\chi$ - κ TNP bearing the chimaeric κ light-chain gene, was transferred as described elsewhere⁷⁻⁹ to the mutant cell line, igk14 (ref. 7). This cell line has lost the ability to produce the TNP-specific κ light chain (κ_{TNP}) but continues to synthesize the TNP-specific μ heavy chain (μ_{TNP}). In a similar manner, the vector pN $\cdot\chi$ - μ TNP bearing the chimaeric heavy-chain gene, was transferred to another mutant cell line, igm10 (ref. 3), which produces κ_{TNP} but no μ_{TNP} . To produce the totally chimaeric IgM, we transferred the vectors pN $\cdot\chi$ - κ TNP and pN $\cdot\chi$ - μ TNP together into the cell line, Sp2/0 (ref. 10), which produces neither heavy nor light immunoglobulin chains. Transformants were selected for resistance to the drug G418, then tested for their production of TNP-specific IgM, as measured by their ability to agglutinate TNP-coupled sheep red blood cells (TNP-SRBC). The frequency at which stable G418-resistant transformants were generated was found to be 10^{-3} . In experiments involving a single vector-transfer, approximately 30% of the resistant transformants produced detectable IgM. In the co-transfer experiment, where the chimaeric κ and μ were introduced into Sp2/0, we estimate that 0.1-1% of the transformants produced enough of both μ_{TNP} and κ_{TNP} to make detectable IgM. The selective advantage to co-transfer seems here to be significantly less than that reported by others using calcium phosphate coprecipitation¹¹ or protoplast fusion¹². We have also transferred the chimaeric heavy- and light-chain genes sequentially: the heavy-chain vector, pN $\cdot\chi$ - μ TNP, was first introduced into

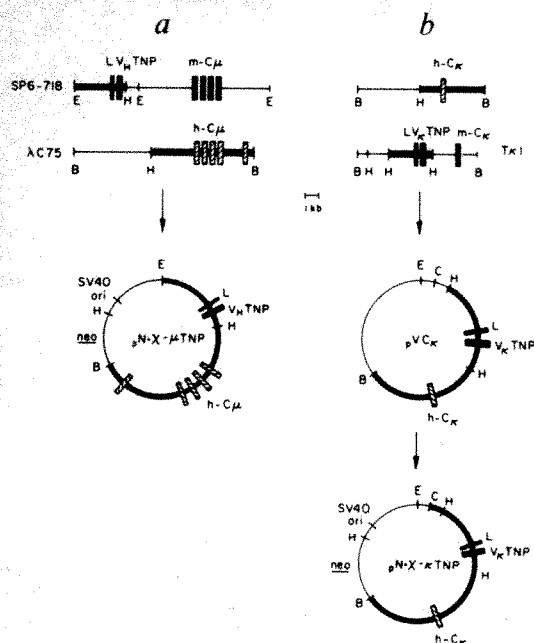


Fig. 1 *a*, Construction of the chimaeric heavy-chain gene. The 3.5-kilobase (kb) *EcoRI*-*HindIII* fragment containing the mouse TNP-specific heavy-chain variable region was obtained from the cloned TNP-specific heavy chain, Sp6-718 (ref. 3). This fragment, together with the 8.0-kb *BamHI*-*HindIII* fragment containing the human constant region from the cloned segment ΔC75 (ref. 4), was introduced into pSV2-neo by ligation at the *EcoRI*-*BamHI* site. *b*, Construction of the chimaeric light-chain gene. The 5.4-kb *BamHI*-*HindIII* fragment containing the human C_{κ} gene was introduced into pVR322 by ligation at the *BamHI*-*HindIII* site. The 4.2-kb *HindIII*-*HindIII* fragment containing the mouse TNP-specific light-chain variable region was obtained from the cloned TNP-specific light chain $T_{\kappa}1$ (ref. 2) and introduced into the *HindIII* site of the pVR322 vector containing the human C_{κ} . From this plasmid, pVC κ , the 9.6-kb *BamHI*-*ClaI* fragment containing the chimaeric light chain was obtained and introduced at the *BamHI*-*ClaI* site of a modified pSV2-neo vector containing the *BamHI*-*EcoRI* segment derived from pVR322. The chimaeric light-chain gene was also introduced into the vector pSV2-gpt¹³ in the manner described above. Restriction enzymes: B, *BamHI*; C, *ClaI*; E, *EcoRI*; H, *HindIII*.

Sp2/0; in a subsequent step, transfer of the light-chain gene, carried in this case on the vector pSV2-gpt¹³, was selected by resistance to mycophenolic acid. Stable transformants which produced the highest levels ($\sim 5 \mu\text{g ml}^{-1}$) of IgM were selected for further study and cloned by limiting dilution. The vector pR-HLTNP, which bears in its entirety the mouse genes for TNP-specific μ and κ chains, was also transferred to the cell line Sp2/0, (refs 3, 14) and the IgM made by one such transformant, TSp2/mIgM12, is compared here with the chimaeric IgM made by the transformant TSp2/ χ -IgM1. By using various antisera specific for antigenic determinants of the mouse and human μ and κ constant regions, we confirmed that the chimaeric genes encode chimaeric proteins, that is, the TNP-binding capacity of the mouse IgM is linked to antigenic determinants of human μ and κ chains (results not shown).

To determine whether the chimaeric IgM is pentameric, we analysed the biosynthetically labelled IgM secreted by these transformants using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-denatured chimaeric IgM from TSp2/ χ -IgM1 migrates at nearly the same rate as pentameric mouse IgM produced by the hybridoma Sp6 and the transformant TSp2/mIgM12 (Fig. 2A), indicating that the chimaeric genes produce μ and κ chains which combine to form pentameric IgM. After reduction of the disulphide bonds, chimaeric μ and κ chains migrate slightly slower than the corresponding mouse chains (Fig. 2B). The molecular weights of the human μ and

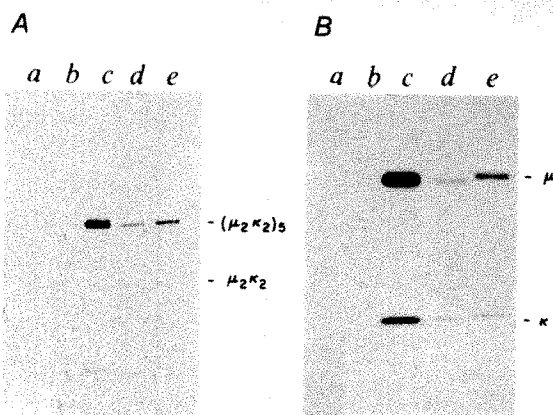


Fig. 2 Production of immunoglobulin heavy and light chains in transformants. The transformants expressing the chimaeric μ and κ genes are compared with two cell lines making TNP-specific murine IgM: the parental Sp6 hybridoma (Sp603 subclone) from which the μ_{TNP} and κ_{TNP} genes were cloned, and a transformant (TSp2/mIgM12) expressing the transferred murine μ_{TNP} and κ_{TNP} genes (denoted as cell line Sp2/T12 in ref. 14). The secreted IgM(κ) of the indicated cell lines was biosynthetically labelled with ¹⁴C-leucine and subjected to SDS-PAGE as described²³. Immunoglobulin from cell lines Sp2/0 (*a*), Sp6 (*c*) and TSp2/mIgM12 (*d*) was precipitated with anti-mouse IgM. Immunoglobulin from the cell lines Sp2/0 (*b*) and TSp2/ χ -IgM1 (*e*) was precipitated with anti-human IgM. *A*, Material was not reduced so that the immunoglobulin disulphide bonds are intact. *B*, Material was treated with 2-mercaptoethanol to reduce disulphide bonds.

κ constant regions are nearly the same as the corresponding murine amino acid sequences¹⁵; therefore the difference in mobility does not reflect simply a difference in molecular weight. Work is in progress to determine the reason for these differences in mobility.

We used two methods to compare the binding sites of mouse and chimaeric IgM. First, we determined the affinity of these IgMs for TNP by measuring the ability of free hapten to inhibit the inactivation by these IgMs of TNP-coupled phage T4 (Fig. 3); the association constant of mouse and chimaeric IgM for the hapten TNP-cap (2,4,6-trinitrophenyl- ϵ -aminocaproic acid) was $1.3 \pm 0.5 \times 10^4 \text{ M}^{-1}$ and $1.5 \pm 0.5 \times 10^4 \text{ M}^{-1}$, respectively. Within experimental error, we can detect no significant difference in affinity for TNP between the mouse and chimaeric IgMs.

We also compared the affinity of these IgMs for each of several trinitrophenyl-like compounds by measuring the ability of these compounds to block the agglutination of TNP-SRBC by IgM. Figure 4 illustrates the results obtained for TNP-cap and DNPy-Ala (3,5-dinitropyridine- β -alanine). The displacement of the inhibition curves indicates that the affinity of each IgM for DNPy-Ala is about threefold less than for TNP-cap. Table 1 summarizes the results obtained for other compounds. Again, these results suggest that the hapten binding sites are comparable in the mouse and chimaeric IgMs. The direct analysis of immunoglobulin structure predicted that immunoglobulin specificity should be unchanged when the same variable region is joined to different constant regions¹⁶. This prediction has been verified both in studies of IgM and IgD in normal cells¹⁷ and in analyses of the binding specificity of the immunoglobulin made by hybridoma cell lines which have switched *in vitro*^{18,19}. In addition, Sharon *et al.*²⁰ have shown that the substitution of a light-chain constant region for a heavy-chain constant region does not affect the affinity of the resulting protein. On the other hand, we can distinguish between the chimaeric and mouse IgMs using hapten inhibition of TNP-SRBC agglutination as a binding assay (Fig. 4). For each hapten, the curve for chimaeric IgM is displaced from the corresponding curve for mouse IgM. The phage inactivation analysis described above indicated that

Table 1 Relative affinity of mouse and chimaeric IgMs for trinitrophenyl-like compounds

Hapten	mIgM	χ -IgM
TNP-cap	1.00	1.00
TNP-Lys	1.00	1.00
DNP-Lys	1.00	1.00
DNP γ -Ala	3.00	3.00
NIP	>10	>25
NP	>10	>25

As described in Fig. 4 legend, we measured the ability of each hapten to inhibit agglutination of TNP-SRBC. TNP-cap was obtained from Dr G. D'Agostura; TNP-Lys (2,4,6-trinitrophenyl-L-lysine) and DNP γ -Ala were obtained from Research Plus; NIP (4-hydroxy-3-iodo-5-nitrophenylacetic acid) and NP (4-hydroxy-3-nitrophenylacetic acid) were obtained from Sigma and Aldrich, respectively. The affinity of each IgM for the indicated haptens, relative to TNP-cap, was determined by the displacement of the inhibition curves. mIgM, murine IgM; χ -IgM, chimaeric IgM.

these IgMs have the same affinity for TNP-cap. In this context, the displacement of the agglutination curves suggests that the binding of the chimaeric IgM to TNP-SRBC is different in some way from that of the mouse IgM. The significance of this difference in molecular terms is unclear. Crystallographic analysis has suggested that there are several sites at which the variable region interacts with the first domain of the constant region¹⁶; such interactions may affect the binding site differently in the chimaeric and mouse IgMs. On the other hand, agglutination is a complex process involving the binding of multiple IgMs at each of the multiple sites. Subtle differences in aspects such as flexibility of the constant region may affect binding and thus the sensitivity to inhibition by free hapten. The molecular stress necessary to effect agglutination may distort the variable region in ways which would not usually occur while the immunoglobulin is free in solution.

We have also compared the chimaeric and mouse IgMs for their ability to activate complement. Culture supernatants were titred for their ability to promote lysis of TNP-SRBC in the presence of a source of complement, that is, guinea pig serum. Compared with the haemagglutination titre, the haemolysis titre for chimaeric IgM was about fourfold less than that for mouse IgM; we do not know whether this reflects a difference in the intrinsic ability of these IgMs to activate complement or the difference in TNP-SRBC binding discussed above.

Several therapeutic uses for specific antibodies have been proposed. The transfer of passive immunity by injecting specific immunoglobulins is a long-standing treatment. Other uses are more speculative. In animal models, anti-idiotypic antibodies have been used both to elicit and to suppress the production of specific antibodies by the recipient animal²¹. These results might be extended to humans so that anti-idiotypic antibodies could be used in some cases as a vaccine to enhance antibody production and in other cases to depress the destructive immune responses which cause autoimmune diseases. The identification of tumour-associated antigens might lead to the production of monoclonal antibodies that selectively destroy tumour cells *in vivo*²². However, as mentioned above, it may prove difficult to obtain human monoclonal antibodies having the specificities required for immunotherapy. Chimaeric immunoglobulins may provide a good compromise. For both monoclonal human immunoglobulin and chimaeric immunoglobulin, the constant region is expected to be non-immunogenic. We have no reason to expect the mouse variable region would in this form be more immunogenic than a human variable region of the same specificity.

In terms of DNA and protein, the chimaeric antibody system works well. The regulatory signals for RNA transcription, initiation, termination and splicing function, so that these genes express a high level of chimaeric μ and κ chains. The specificity of the mouse variable region is preserved in the chimaeric IgM, and the human constant regions function so that the μ and κ

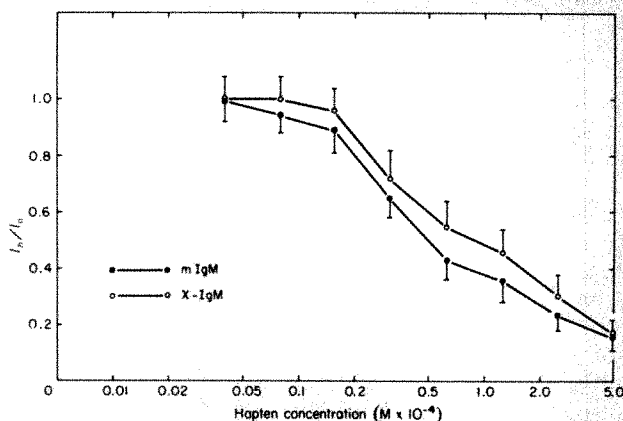


Fig. 3 Affinity of chimaeric and mouse IgMs for TNP. TNP was coupled to phage T4 as described elsewhere²⁴. The affinity for hapten was calculated as follows. The rate-limiting step for the inactivation of phage T4 is the attachment of the first immunoglobulin binding site²⁵, that is, the concentration of surviving phage, Φ , is given by $\Phi = P \exp(-aB)$ where P = initial haptenated phage concentration before reacting with the anti-TNP, B = concentration of free binding sites and a = proportionality constant. By incubating free hapten in the reaction mix, the concentration of free binding sites can be manipulated according to the formula $K_a = [hB]/[h][B]$, where K_a is the association constant, $[h]$ the hapten concentration, $[B]$ the concentration of free binding sites and $[hB]$ the concentration of binding sites bound to hapten. The inactivation index, I_h , is defined as $\log \Phi(h)/P$ where $\Phi(h)$ is the phage concentration after incubation with anti-TNP in the presence of hapten at concentration h . The figure plots the ratio I_h/I_0 as a function of hapten concentration where I_0 is the inactivation index obtained in the absence of free hapten ($h=0$). Each point was determined in triplicate. The affinity constant for the hapten can be calculated from any point as $K_a = 1/h[I_0/I_h - 1]$. The values of K_a given in the text represent the average of all points.

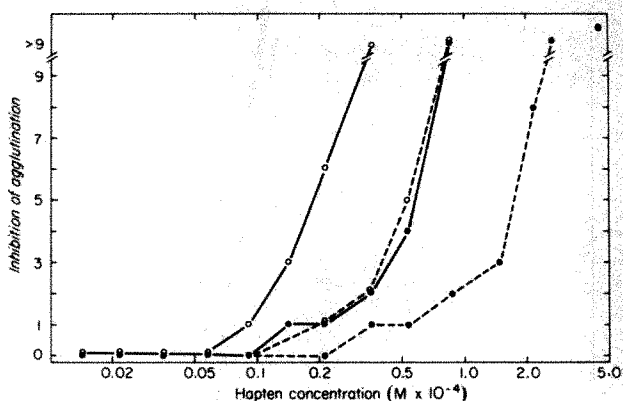


Fig. 4 Relative affinity of mIgM (●) and χ -IgM (○) for TNP (—) and DNP γ -Ala (---) haptens. Culture supernatants from the transformants TSp2/mIgM12 and TSp2/ χ -IgM1 were serially diluted in twofold steps and incubated with either TNP-cap or DNP γ -Ala at the indicated concentration in V-bottomed 96-well trays. TNP-SRBC were then added and the wells were scored for agglutination. The inhibition of agglutination, that is, the reduction in the number of wells with positive haemagglutination, is shown here for the indicated concentrations of each hapten. The displacement of the curves for the different haptens indicates the hapten concentrations required to yield the equivalent number of free binding sites, and thus measures the relative affinities of each IgM for the two haptens²⁶.

chains are covalently bound to form pentameric IgM which can activate complement. We have, nevertheless, detected differences in the binding to TNP-SRBC of the chimaeric and mouse IgMs. Further work is needed to assess whether these differences have significance for immunotherapy.

We acknowledge important contributions by Dr C. Heusser in the conception of this work. This work was supported by

grants from the MRC of Canada, the NCI of Canada, the Arthritis Society of Canada, the Wellesley Hospital Research Institute and the Allstate Foundation. G.L.B. is supported by a studentship of the NCI of Canada.
ed 5 September 1984.

Received 2 July; accepted 5 September 1984.

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Participation of p53 cellular tumour antigen in transformation of normal embryonic cells

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The cellular tumour antigen p53 is found at elevated levels in a wide variety of transformed cells (for reviews see refs 1, 2). Very little is yet known about the precise relationship of p53 to malignant transformation. Although the increase in p53 levels could be a secondary by-product of the transformed state, it is equally possible that p53 is actively involved in altering cellular growth properties, especially as it has been implicated in the regulation of normal cell proliferation³⁻⁶. We sought to test whether p53 could behave in a manner similar to known genes in a biological test system, and we demonstrate here that p53 can cooperate with the activated Ha-ras oncogene to transform normal embryonic cells. The resultant foci contain cells of a markedly altered morphology which produce high levels of p53. Cell lines established from such foci elicit tumours in syngeneic animals.

Recent findings have suggested certain similarities between p53 and the product of the oncogene *myc*. Both are DNA binding proteins (ref. 7 and D. Lane, personal communication) that accumulate in the nuclei of transformed cells^{7,8}. Both are regulated with the cell cycle^{6,9,10} and are induced at an early stage following the treatment of resting cells with mitogens^{3,5,9,10}. Cycloheximide-treated cells accumulate p53¹¹ and show increased *myc* messenger RNA levels⁹. Detailed analysis of the amino acid sequences predicted for the two proteins shows weak similarities in both the overall molecular organization and the positioning of charged residues within distinct domains of the molecules^{12,13}. Hence, if p53 can function like known oncogenes, it is likely to do so in a manner similar to *myc*.

Table 1 Transformation of rat and Chinese hamster embryo fibroblasts by various gene combinations

Transfected DNA	Foci per 10 ⁶ cells			Tumorigenicity
	REF expt 1	REF expt 2	CHEF expt 3	
Carrier				
(BALB/c DNA)	0	0	0	
pEJ6.6	0	0	0	
pMSVp53G	0	0	ND	
pPyp53c	ND	ND	0	
pMSVp53G + pEJ6.6	13	5	ND	15/15
pPyp53c + pEJ6.6	ND	ND	20	
pLSVmyc + pEJ6.6	ND	21	ND	9/9
pLA8 + pEJ6.6	68*	ND	165*	
pMSVE + pEJ6.6	ND	0	ND	
None				0/8

Primary Fisher rat or Chinese hamster embryo fibroblasts were prepared³⁰ and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 4 mM L-glutamine (maintenance medium). 10⁶ cells were seeded per 90-mm dish, and were transfected with the indicated DNA combination after 1 day (10 µg of each plasmid, made up to a total of 25 µg with sheared BALB/c liver DNA). pLSVmyc was constructed linking the 5.6 kb *Bam*HI fragment of the murine *c-myc* gene (see ref. 14) to the SV40 early promoter. pLA8 contains the left-end 9.1% of the adenovirus12 genome, including the EIA and part of the E1B region¹⁶. pMSVE is a derivative of pMSVp53G, containing only the MSV enhancer inserted in the *Bam*HI site but no p53-specific sequences (compare with Fig. 1). The cells were transfected by the calcium phosphate procedure³¹ for 16 h, glycerol-shocked (10% glycerol in maintenance medium for 90 s), replenished with maintenance medium, allowed to recover for an additional day, then split and reseeded at a density of 4 × 10⁵ (rat fibroblasts) or 2 × 10⁵ (Chinese hamster cells) per 90-mm dish. Medium was changed every 6-7 days. Distinct foci overgrowing the monolayer were scored 16 days (expt 1), 14 days (expt 2) or 24 days (expt 3) after replating of transfected cultures. REF, rat embryo fibroblasts; CHEF, Chinese hamster embryo fibroblasts. The tumorigenicity of cell lines established from corresponding foci was determined by injecting subcutaneously 5 × 10⁶ cells into 5-8 day-old Fisher rats whole-body irradiated with 200 rads. As a non-transfected control (bottom line) we used REF propagated in culture to obtain a sufficient number of cells. Data are derived from two different p53 + Ha-ras lines and one *myc* + Ha-ras line. ND, not determined.

* Foci possessing a distinctly different morphology from that induced by pLA8 alone.

A biological test system demonstrating the involvement of the *myc* product in malignant transformation has been established recently^{14,15}; primary rat embryo fibroblasts are transformed stably by the joint action of *myc* and another oncogene such as Ha-ras. Similar results are obtained when another nuclear oncogene, the adenovirus-2 E1A region, is assayed by co-transfection with Ha-ras in an analogous system¹⁶. In both cases, the transformation is visualized by the appearance of dense foci capable of overgrowing the monolayer of normal cells and are dependent on the presence of both oncogenes. This system is therefore a suitable test of the oncogenic properties of p53.

Two types of recombinant DNA constructs were used as templates for efficient p53 expression in transfected cells (Fig. 1). The plasmid pMSVp53G contains the 16 kilobase (kb) *Eco*RI fragment encompassing the functional murine p53 gene^{12,17} juxtaposed to the enhancer portion of the Moloney murine sarcoma virus (MoMSV) long terminal repeat. This approach utilizes the presence of a functional promoter in the 16 kb fragment (B. Bienz, unpublished results). The second construct, pPyp53c, contains a stretch of p53 cDNA linked to the polyoma virus early promoter; this cDNA contains the intact coding region for p53¹⁷ and directs the synthesis of authentic p53 in a heterologous system¹⁸.

Secondary Fisher rat embryo fibroblasts were co-transfected with pMSVp53G and pEJ6.6 (ref. 19), carrying an activated human *c-Ha-ras*1 gene. As a positive control, we performed a

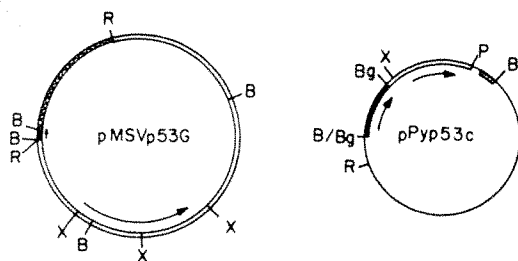


Fig. 1 p53-specific plasmids used for transfection. pMSVp53G contains the functional mouse p53 gene linked to the enhancer element of MoMSV DNA. The 16 kb *EcoRI* fragment was excised out of the recombinant phage Ch 53-7 (ref. 12) and inserted into the *EcoRI* site of plasmid pA₁₀ (ref. 32). The *HinfI*-*XbaI* fragment of the MoMSV long terminal repeat, containing the enhancer portion³³, was converted at both ends to *BamHI* and introduced into the *BamHI* site of the same pA₁₀ plasmid. Hatched bar, pA₁₀ DNA; full bar, MoMSV DNA; open bar, p53-specific genomic DNA. To construct pPyp53c, the *BamHI*-*HphI* fragment of polyoma (Py) DNA, containing the early promoter³⁴, was converted to *BglII* at both ends. This was inserted into pSVp53c17 DNA¹⁸, cleaved previously with *BglII* and partially cleaved with *BamHI* to remove the bulk of SV40 sequences. Briefly, pPyp53c consists of the following components (from 5' to 3') inserted within the *BamHI* site of pBR322: first, the Py early promoter region, a segment of p53 cDNA extending from the *BanI* site at nucleotide -67 (ref. 17) to the end of the insert of pp53-176 (nucleotide 1,188, ref. 17) and including also 25 base pairs of oligo(dG):oligo(dC); second, 125 base pairs of pBR322 DNA corresponding to the region between the *PstI* site and the nearby *BglII* site³⁵; finally, the *BclI*-*BamHI* fragment of SV40 DNA, derived from plasmid pLSV and including the viral early polyadenylation site³⁶. Thin line, pBR322 sequences; full bar, Py DNA; open bar, p53 cDNA; hatched bar, SV40 DNA, B, *BamHI*; Bg, *BglII*; P, *PstI*; R, *EcoRI*; X, *XhoI*. For clarity, only the *PstI* site originally harbouring the cDNA insert of clone pp53-176 (ref. 17) is indicated. Arrows, normal transcriptional orientation.

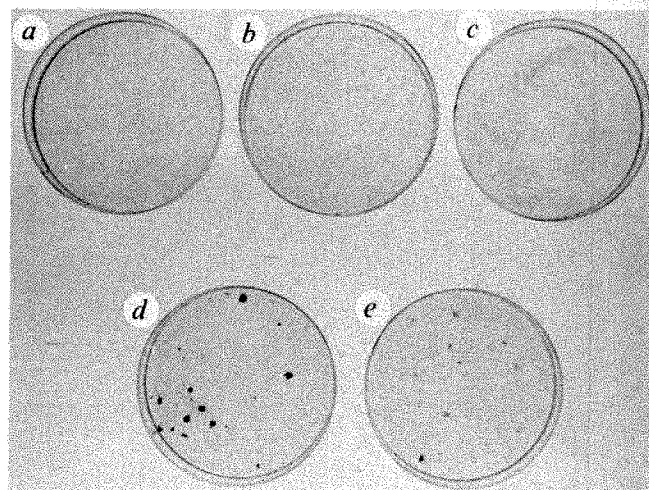


Fig. 2 Giemsa-stained rat embryo fibroblast cultures transfected with various DNA combinations. Transfection was as described in Table 1, using the following DNA: a, BALB/c carrier DNA alone; b, pEJ6.6 + carrier; c, pMSVp53G + carrier; d, pLA8 + pEJ6.6 carrier; e, pMSVp53G + pEJ6.6 + carrier. Photographs were taken 16 days after replating of transfected cells.

co-transfection with pEJ6.6 and pLSVmyc. The latter plasmid contains the protein-coding part of the mouse *myc* gene, driven by the simian virus 40 (SV40) early promoter (see Table 1). In some cases, pLSVmyc was replaced by pLA8 (ref. 16), containing the left end 9.1% of the adenovirus-2 genome.

We found that although neither p53 nor Ha-*ras* alone induced the appearance of foci, their joint action could cause this effect (Table 1). The foci generated had a distinct morphology (Fig. 2) and could usually be detected after 9–12 days. In these experiments, p53 was less efficient than *myc* and markedly less efficient than pLA8. Although pLA8 by itself is sufficient for transformation¹⁶, the foci induced by its co-transfection with pEJ6.6 grew much more rapidly than those due to pLA8 alone and had a very different, easily recognizable morphology (data not shown).

To test that the observed co-transformation is not due to activation of unrelated genes by the enhancer in pMSVp53G^{20,21}, the latter plasmid was substituted by an analogue lacking p53 sequences; no foci were generated (Table 1).

The morphology of the foci cells varied with the particular gene combination, although all were clearly distinct from the surrounding non-transformed cells. Cells transformed by p53 + Ha-*ras* (Fig. 3g) were large, refractile and more similar to the *myc* + Ha-*ras* transformants (Fig. 3e), whereas the foci arising in monolayers transfected with pLA8 + Ha-*ras* were composed mostly of smaller cells (Fig. 3c). p53 + Ha-*ras* foci tended to grow more slowly than *myc* + Ha-*ras* foci and often appeared to stop spreading after reaching 5–7 mm diameter, whereas *myc* + Ha-*ras* foci grew continuously until they merged and filled the dish.

Similar results were obtained using an alternative system, secondary Chinese hamster embryo fibroblasts and the cDNA construct pPyp53c (Table 1). Co-transfection with p53 and pEJ6.6 resulted in overgrowing foci of cells with transformed

morphology (Fig. 3h,i). However, these experiments were hampered because the transformed cells were prone to lysis.

If p53 production is involved in the observed transformation, the protein should be made in the transformed cells. Accordingly, cells were isolated from foci, labelled with ³⁵S-methionine and assayed for p53. We used anti-p53 monoclonal antibody RA3-2C2 (refs 22, 23); this is specific for the murine form of the protein and does not cross-react with rat p53 (ref. 24), thus allowing the detection of transfection-derived murine p53 in the transformed rat cells and avoiding potential difficulties due to the presence of endogenous rat p53. Murine p53 could be immunoprecipitated easily from overgrowing foci cells (Fig. 4), sometimes in even higher concentrations than those present in Meth A mouse fibrosarcoma cells, marked overproducers of p53 (ref. 25). This suggests strongly that the actual production of the protein is involved in the transformation.

Unlike *myc* + Ha-*ras* or pLA8 + Ha-*ras*, it proved difficult to establish cell lines from foci induced by p53 + Ha-*ras*. Typical transformed cells exhibited a limited proliferation potential and, after repeated passaging, cultures became dominated by cells of a normal-nontransformed morphology, probably derived from the underlying monolayer. The level of murine p53 fell simultaneously to below detection after two or three passages (data not shown). These findings support the notion that p53 production, directed by the transfected DNA, is involved in the observed transformation. Almost all cells of distinct transformed morphology, when transferred into a new dish, attached to it but did not divide. On the other hand, the few normal cells transferred with the focus did start dividing once replated at a low density. Eventually, with several low-density passages, replicating normal cells became predominant. These data suggest that, unlike *myc* or EIA^{26,27}, p53 is not capable of efficiently immortalizing cells. Thus, the p53 + Ha-*ras* transfectants, although transformed, still undergo a senescence crisis and stop multiplying. The normal underlying cells, however, are still able to resume replication once subcultured at a low density, hence they can easily overgrow the senescent transformed cells. Nevertheless, a very small proportion of transformed cells did eventually give rise to stable transformed cell lines. Thus, when foci were subcultured and cells maintained in the dish for 10–14 days without further passaging, a few overgrowing foci became evident. The cells comprising these secondary foci, while generally morphologically indistinguishable from those found in the original foci, did possess an apparently unlimited growth

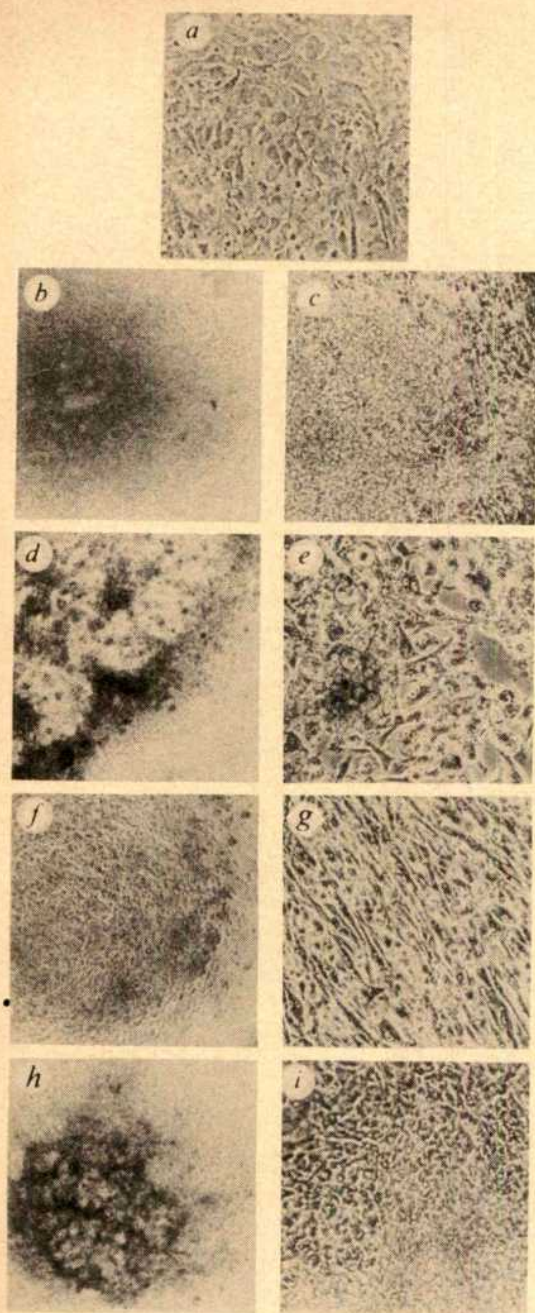


Fig. 3 Morphology of cells transformed by various plasmid combinations. Pictures were taken at two magnifications to display the typical appearance both of the foci and of individual cells within them. *a*, REF (Table 1) transfected with carrier only; *b, c*, REF transfected with pLA8+pEJ6.6; *d, e*, REF transfected with pLSVmyc+pEJ6.6; *f, g*, REF transfected with pMSVp53G+pEJ6.6; *h, i*, CHEF transfected with pPyp53c+pEJ6.6. Normal CHEF are visible in the upper left corner of *i*. Magnification: *b, d, f, h*, $\times 40$; *a, c, e, g, i*, $\times 100$.

potential and could be propagated easily to yield homogeneous stably-transformed cell lines. When two such lines were subjected to more detailed analysis, both expressed high levels of murine p53 (data not shown). On injection into syngeneic young rats, both cell lines generated tumours at a high efficiency (Table 1) which developed significantly more slowly than those induced by pLSVmyc + EJ6.6 transformants. In some animals, they stopped growing after reaching 6–10 mm diameter, whereas in most cases they grew continuously, reaching a very large size and eventually killing the animal. We are currently testing more lines

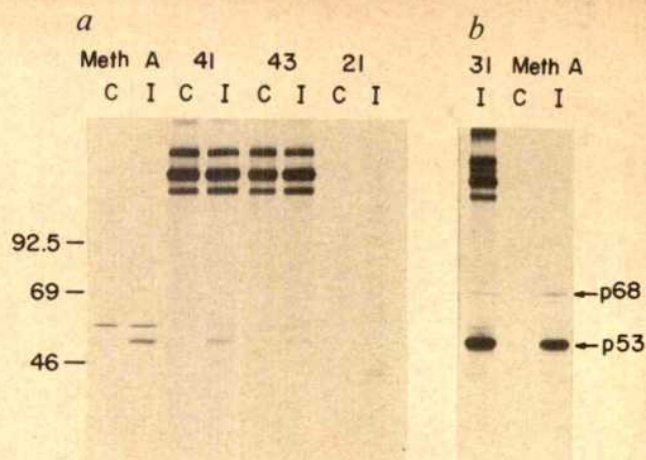


Fig. 4 Analysis of proteins produced in focus-derived rat cells. Foci arising in a monolayer of rat embryo fibroblasts following transfection with different DNA combinations were trypsinized, expanded into 35-mm Petri dish cultures and labelled with $40 \mu\text{Ci}$ ^{35}S -methionine for 4 h. Extracts were prepared as described previously³⁷. Equal amounts of trichloroacetic acid-insoluble radioactivity (2.8×10^6 c.p.m. per lane) were immunoprecipitated with either anti-mouse p53 monoclonal antibody RA3-2C2²²⁻²⁴ or control serum, using standard procedures³⁸, followed by electrophoresis through a 12.5% SDS-polyacrylamide gel³⁸. *a, b* refer to two separate experiments. *a*, Autoradiography for 2 days; *b*, for 7 days. Meth A are chemically transformed mouse fibroblasts which overproduce p53 (ref. 25); 41, 43 and 31 are derived from foci induced by pMSVp53G+pEJ6.6 while 21 is derived from a focus induced by pLA8+pEJ6.6. C, control serum, I, RA3-2C2 monoclonal antibody. Numbers on the left refer to the relative molecular mass ($\times 10^{-3}$) of co-electrophoresed markers. p68 is a polypeptide co-precipitating with mouse p53 (see ref. 18).

to determine whether this is a general feature of p53 + Ha-ras transformants.

Based on the results presented here and in ref. 28, we conclude that p53 can act as a nuclear oncogene by collaborating with Ha-ras in the transformation of cultured embryonic fibroblasts. Unlike *myc* or EIA, only a small proportion of the resultant transformants eventually become immortalized and give rise to stable lines, possibly through a change involving a third gene product in addition to p53 and Ha-ras. The initial transformed cells must be highly predisposed towards this activation event, as it occurs much more frequently than predicted for random mutations. Alternatively, the few immortalized cells may be selected for their ability to produce increased amounts of either p53 or Ha-ras protein. Recent data²⁹ suggest that massive overproduction of the Ha-ras p21 protein can circumvent the need for a cooperating oncogene. The rare alteration leading to immortalization may thus be quantitative rather than qualitative. Immortalization may not be a general attribute of nuclear transforming proteins. Although *myc* and EIA can efficiently immortalize cells^{26,27}, their ability to induce overgrowing foci in combination with Ha-ras may reflect an entirely different facet of their activity, the two oncogenes sharing only certain properties with p53.

We thank O. Pinhasi and R. Karkash for technical assistance, S. Lavi, S. Etkin and M. Horowitz for suggestions, Y. Gluzman, M. Horowitz and H. Land for gifts of pLA8, pLSVmyc and pEJ6.6, respectively, and R. A. Weinberg for communication of results before publication. This work was supported by grants from the Leukaemia Research Foundation, Inc., the Fund for Basic Research of the Israeli Academy of Sciences and Humanities and the Bat-Sheva de Rothschild Fund for the Advancement of Science and Technology. M.O. is a Cancer Research Scientist supported by the Rose and George Blumenthal Fellowship of the Israel Cancer Research Fund.

Received 3 July; accepted 13 September 1984.

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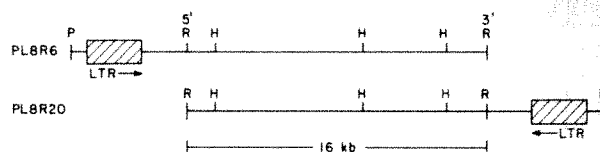


Fig. 1 Schematic diagram of *p53* encoding plasmids. (For detailed description, see ref. 15.) Briefly, the 16kb mouse DNA fragment containing all of the *p53*-encoding exons was inserted either downstream from and in the same transcriptional orientation as the MuLV long terminal repeat (PL8R6), or upstream and in the opposite transcriptional orientation (PL8R20). The vector is pBR322. R, *EcoRI* restriction sites; H, *HindIII* restriction sites; P, *PstI* restriction sites.

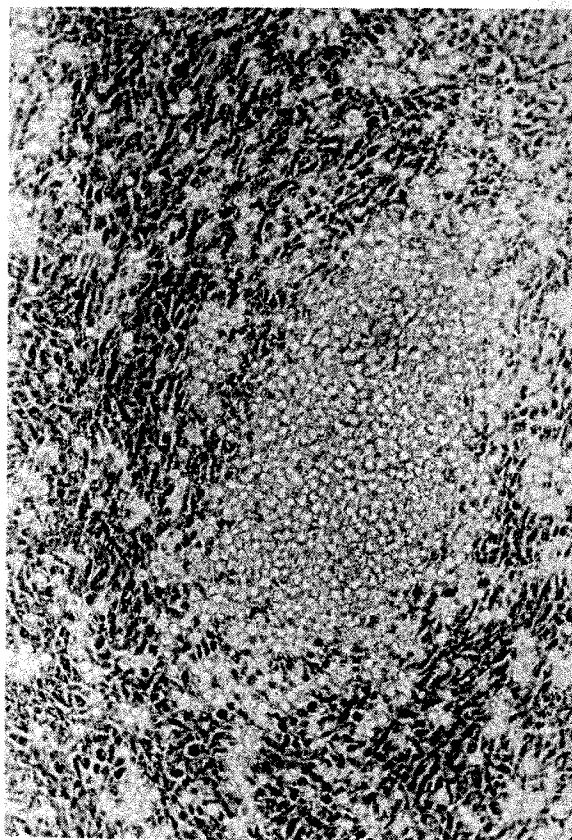


Fig. 2 Phase contrast photomicrograph of a focus arising in a dense monolayer of normal rat embryo fibroblasts after co-transfection of PL8R20 and the EJ-*ras* oncogene. The DNA transfection was performed as described in Table 1. The photograph was taken 10 days after transfection.

Cooperation between gene encoding *p53* tumour antigen and *ras* in cellular transformation

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The protein *p53* is highly expressed in a large variety of transformed cell types originating from diverse species. These include cells transformed by Simian virus 40 (SV40), adenovirus and Abelson virus, as well as a variety of chemically transformed cells¹⁻⁶. Substantial amounts of *p53* are also present in certain non-transformed cells, for example, some embryonic tissues⁷. The protein may be localized in different cellular compartments in normal and transformed cells⁸. The strong correlation between tumorigenicity and high levels of *p53* suggests an important role of *p53* in tumorigenesis. We report here experiments in which we have co-transfected the murine cellular gene encoding for *p53* with a *ras* gene into primary rat embryo fibroblasts. Our results indicate that the *p53*-encoding gene can play a causal role in the conversion of normal fibroblasts into tumorigenic cells.

A cDNA clone of the *p53* gene^{9,10} was used to retrieve a homologous 16 kilobase *EcoRI* segment from a genomic library made from DNA of a murine B-cell lymphoma induced by Abelson virus¹¹. This genomic clone was modified by linkage to the murine leukemia virus (MLV) promoter-enhancer long terminal repeat element. These long-terminal repeat sequences were inserted to induce a high level of expression of the cloned *p53* gene (Fig. 1).

We performed transfection experiments with clones of the modified genes (see Fig. 1) to determine whether these clones

would induce foci in monolayer cultures of the Rat-1 fibroblast cell line or in cultures of secondary rat embryo fibroblasts (REFs). All transfections include the pSV2neo³³ plasmid DNA so that a portion of the cultures could be placed under G418 drug selection to control for transfection efficiencies.

The various *p53* clones tested are unable to induce foci in any of the transfected Rat-1 cultures, even when such cultures are surveyed weeks after transfection (Table 1). In the same experiments, a *ras* oncogene elicits foci observable in Rat-1 cells within eight days of transfection. We conclude that the *p53* gene, unlike the *ras* oncogenes, has no apparent ability to transform readily established rodent fibroblast cells.

Earlier work had shown that a single oncogene, such as *ras* or *myc*, is unable to induce foci in REF monolayer cultures. However, when the two oncogenes are applied together, foci are induced which contain cells that are tumorigenic in young rats or nude mice^{12,13}. The observed inability of *ras* or *p53* clones to yield foci in transfected REF cultures (Table 1) is consistent with these earlier results.

Table 1 Transforming of REFs and Rat-1 cells after co-transfection of the p53-encoding gene and the EJ-c-Ha-ras oncogene

Transfected gene	No of foci per 10 ⁵ cells		Tumorigenicity of REFs nude mice (No. of tumours/no. of injections)
	REFs	RAT-1	
REF/DNA	0	0	0/10
pEJ6.6	0	2,000-2,500	0/20
pSVcmv-1	0	0	0/15
pEJ6.6 + pSVcmv-1	200-300	2,000-2,500	†29/29
pL8R6	0	0	0/5
pL8R20	0	0	ND
pL8R6 + pEJ6.6	20-30	2,000-2,500	‡6/6
pL8R20 + pEJ6.6	10-20	2,000-2,500	§3/3
pL8R6 + pSVcmv-1	0	0	0/6
pL8R20 + pSVcmv-1	0	0	ND
*pL8R6 (<i>Bam</i> HI/ <i>Hind</i> III + pEJ6.6)	0	ND	0/3

The preparation of primary cultures of Fisher REFs the transfection procedures and plasmids pEJ6.6 and pSVc-myc1 are described elsewhere¹². 0.5 µg of PSV2neo³³ DNA per 2 × 10⁶ cells was included in all transfections. Subcutaneous injections to test for tumorigenicity made into 30-40-day-old nude mice irradiated (500 rad) 24 h before. All injected mice were observed for five weeks. ND, not determined.

* pL8R6 digested to completion with restriction endonucleases *Bam*HI and *Hind*III to inactivate the p53 gene but not the MLV-LTR promoter-enhancer. At 6 weeks, one of these mice exhibited a small tumour. The nature of this tumour is under study.

† Tumours detected 10-14 days after injection.

‡ Tumours detected 15-20 days after injection.

§ Tumours detected 20-25 days after injection.

The previously observed cooperation of *ras* and *myc* oncogenes suggests an experimental test by which the oncogenic function of other genes can be assayed: a DNA clone carrying a gene of interest is co-transfected with either a *ras* or a *myc* clone and the appearance of REF foci then scored. Accordingly, we tested the two p53 gene clones for cooperation with *ras* or *myc* oncogenes in the transformation of REFs. Neither of the two p53 constructions induce foci when co-transfected with a *myc* oncogene clone. However, when these p53 constructions were tested in conjunction with the human EJ/T24 Ha-*ras* oncogene, they do cooperate in focus induction. These *ras*-p53 foci appear with the same kinetics as *ras*-*myc* foci, but with 10-fold lower frequency. The *ras*-p53 foci are quite large, containing very refractile and rounded cells which do not adhere well to the culture dish (Fig. 2).

We next attempted to demonstrate that the entire p53 chimeric gene, and not simply attached promoter-enhancer sequences, cooperate with the *ras* gene in transformation. The p53 clone pL8R6, which is active in the co-transfection assay, was digested to completion with the restriction endonucleases *Bam*HI and *Hind*III. These enzymes cut the p53 domain into several fragments but leave the linked long-terminal repeat promoter-enhancer segment intact. Co-transfections of the digested clone and the *ras* oncogene do not yield any foci in REFs. We conclude that the sequences within the p53 gene itself are required to cooperate with *ras* in transforming REFs *in vitro*. A second type of promoter-enhancer, introduced at the 3' end of the p53 gene, is unable to adequately activate the gene (data not shown). This and other evidence (ref. 11; D.W. & V.R., unpublished observations), suggests that appropriate transcriptional activation of the p53 clone is required for biological activity.

A more stringent test for transformation than that of focus induction is the assay for tumorigenicity. Fourteen days after transfection, we injected nude mice with the cells from transfected cultures. Only a small proportion of cells from such cultures have acquired and expressed the transfected oncogenes; such transfected cells have not had time to undergo extensive additional alterations, such as may occur during isolation of clonal lines before injection. These injected cultures include the doubly transfected cultures *ras* + *myc* or *ras* + p53, as well as those singly transfected with either *ras* or *myc* or p53 clones (Table 1). Ten days after injection, tumours appear at all the sites injected with *ras* + *myc* transfectants, whereas only small nodules are visible at the sites carrying *ras* + p53 cells. After 18 days, however, all sites injected with *ras* + p53 transfectants exhibit large, aggressively growing tumours.

Cultures carrying *myc* + p53 co-transfected cells develop no foci and yield no tumours upon injection. Thus it appears that p53 is able to function analogously to *myc* by cooperating with

ras oncogenes to induce tumorigenic conversion of rat embryo fibroblasts. The correlation was absolute between the ability of co-transfected cloned DNAs to induce foci in REF monolayers and the ability of these DNAs to yield tumorigenic cultures.

The delayed appearance of *ras* + p53 tumours may be due to the smaller initial number of transformed cells present in the inocula. *Ras* + *myc* transfected dishes contain on average 50 foci. Consequently, each inoculum of 10⁶ cells contains about 2.5 × 10⁴ transformed cells. *Ras* + p53 dishes contain approximately five foci and only about 2,500 transformed cells present among the normal rat embryo fibroblasts. Although delayed in appearance, the tumours stemming from these *ras* + p53 transfectants rapidly reach the size of *ras* + *myc* tumours. All tumorigenic lines resulting from *ras* + p53 co-transfection carried intact, acquired p53 DNA segments (data not shown).

The existence of a p53-specific monoclonal antibody, RA3-2C2^{14,15}, facilitates the protein analysis of the p53 transfectants. This antibody is specifically reactive only with mouse p53¹⁵. The p53 DNA clones used here were isolated from DNA of the Abelson virus-transformed, mouse lymphoid line 230-23-8 (ref. 11). This antibody allows the specific detection of p53 expressed by introduced DNA clones because it has no cross-reactivity with the endogenous, homologous p53 protein of the rat host cell. All transfectants demonstrate the presence of a mouse p53 band, although at differing levels (Fig. 3). This suggests that the continued presence of p53 is required to maintain the transformed phenotype.

The results presented here and in the accompanying paper of Eliyahu *et al.*¹⁶, demonstrate that a modified p53 gene can function as an active oncogene that contributes to tumorigenic conversion. We conclude that expression of p53 can be more than a consequence of the cancer state. Instead, it appears that an active p53 gene can play a causal role in tumorigenicity and can be classified as an oncogene. The p53 oncogenic functions are analogous to cellular *myc* and *myb* oncogenes. (H.L. *et al.*, manuscript in preparation). Although weaker than *myc* in its ability to cooperate with *ras*, we predict that the two genes, p53 and *myc*, will be shown eventually to express many similar functions.

In addition to the functional analogies between p53 and *myc*, some biochemical similarities are apparent. Both genes encode nuclear proteins¹⁷⁻¹⁹. In nontransformed cells, these proteins are metabolically highly unstable, with half lives of less than 60 min (refs 20, 21; R. Eisenmann, personal communication). Both the proteins are phosphorylated²²⁻²⁴ and their expression is linked strongly to the growth state of the cell. In both cases, large increases in expression can be observed following serum stimulation of quiescent cells^{25,26}. This increase of p53 expression seems to be required for induction of DNA replica-

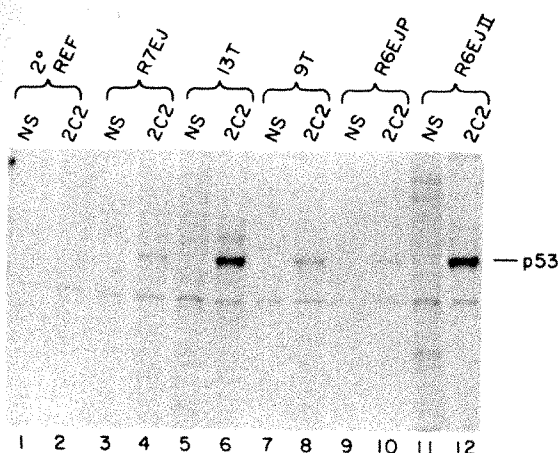


Fig. 3 Analysis of p53 protein cell lines and tumours derived from rat embryo fibroblasts co-transfected with the p53 gene and the EJ-ras oncogene. Cells in 60 mm dishes were labelled for 2 h with 100 μ Ci [^{35} S]-methionine in dialysed calf serum and methionine-free Dulbecco's Modified Eagles medium. Lysates were prepared as described previously¹⁵ and incubated for 1 h at 4°C either with normal rat serum (lanes 1, 3, 5, 7, 9, 11) or with monoclonal antibody RA3-2C2^{20,21} (lanes 2, 4, 6, 8, 10, 12). Protein A Sepharose was added and the lysates incubated at 4°C in a rocking bath for 1 h. Pellets were washed 5 times with RIPA buffer, boiled for 3 min and loaded onto a 10% Laemmli gel³², run at 150 V for 5 h. Lanes 1, 2, secondary rat embryo fibroblasts which have not been transfected; lanes 3, 4, R7EJ, a transformed rat embryo fibroblast cell line derived from co-transfection of the EJ-ras oncogene and PL8R7 (vector not described here), a p53 gene with an SV40 promoter; lanes 5, 6, 13T, a cell line prepared from an explanted nude mouse tumour created by injection of the cell line R7EJ; lanes 7, 8, 9T, a cell line prepared from an explanted nude mouse tumour created by injection of the R6EJP cell line; lanes 9, 10, R6EJP, a transformed cell line derived from co-transfection of PL8R6 and the EJ-ras oncogene; lanes 11, 12, R6EJII, an independently isolated transformed rat embryo fibroblast cell line derived from transfection of PL8R6 and the EJ-ras oncogene.

tion. Microinjection experiments have shown that monoclonal antibodies to p53 can inhibit serum-induced DNA synthesis in 3T3 cells^{27,28}.

SV40 and adenovirus both encode proteins that bind to p53^{4,29}. In the case of the SV40 protein, this ability to bind to p53 appears to increase its half life to 24 h^{20,21}. From the results presented here, it seems plausible that part of the physiological effects of the SV40 large T antigen, including its ability to immortalize cells, may depend on its ability to stabilize p53 and thus increase its effective concentration. We surmise that deregulation of p53 expression can be achieved at the transcriptional level as well. By linking the p53 gene to strong promoter-enhancers, we have created a functional state which is analogous to linking of the normal *myc* gene to integrated retrovirus genomes³⁰, or immunoglobulin genes³¹. It remains to be determined whether this transcriptional deregulation is sufficient to create oncogenic activation, or whether additional, undiscovered lesions in the protein-encoding portion of the gene must be present also for an active oncogene to arise.

We thank Anne Chen and Jay Morgenstern for technical assistance, Bernard Mathey-Prevot and Peter Sarnow for helpful discussions and Moshe Oren for informative discussions in advance of his publication. This work was supported by Grant CA 26717 from the USNCI and by a grant from the American Business Cancer Foundation. H.L. was a fellow of the Deutsche Forschungsgemeinschaft.

Received 23 July; accepted 2 October 1984.

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Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53

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Malignant transformation of primary cells requires at least two distinct and characteristic alterations in cellular behaviour¹. The first, cellular immortality^{1,2}, can be induced by chemical carcinogens³ or by cloned oncogenes such as polyoma large T (ref. 4), adenovirus early region 1A (E1A) or the oncogene from avian (MC29) myelocytomatosis virus, *v-myc*. Cells whose *in vitro* life-span has been extended by these procedures can be fully transformed by transfection with oncogenes belonging to a different complementation group, including genes of the *ras* family, adenovirus E1b and polyoma virus middle T (refs 4, 5). The unstable cellular phosphoprotein p53 is frequently present at elevated levels in transformed cells⁶⁻¹¹ and is stabilized by the formation of complexes with simian virus 40 (SV40) large T or adenovirus E1b 57K protein¹⁰⁻¹³. Although several reports have associated p53 with cell proliferation¹⁴⁻¹⁷, its role remains obscure. We have cloned complementary DNA sequences encoding murine p53¹⁸ and report here that transfection of p53 expression constructs into cells of finite lifespan *in vitro* results in cellular immortality and susceptibility to transformation by a *ras* oncogene.

Adult rat chondrocytes obtained from the xiphisternum undergo ~30 doublings *in vitro* before senescence, with characteristic changes in morphology and cessation of growth. Wistar adult xiphisternum chondrocytes (WAXI) at passage 5 grow slowly, with a doubling time of >60 h. Because spontaneous immortal colonies appear very rarely in such cultures, we have used them in an immortalization assay rather than rat embryo fibroblasts, which we have found to show an irregular and unpredictable pattern of senescence coupled with an unacceptably high incidence of spontaneous immortalization. Furthermore, such cultures transfected with the p53 expression vectors provided a low incidence of immortalization (<2 foci per μ g DNA per 10^6 cells).

We used three p53 expression vectors, the construction and properties of which will be described elsewhere. The standard calcium phosphate co-precipitation method¹⁹ was used to introduce constructs into WAXI cells. In the first round of experi-

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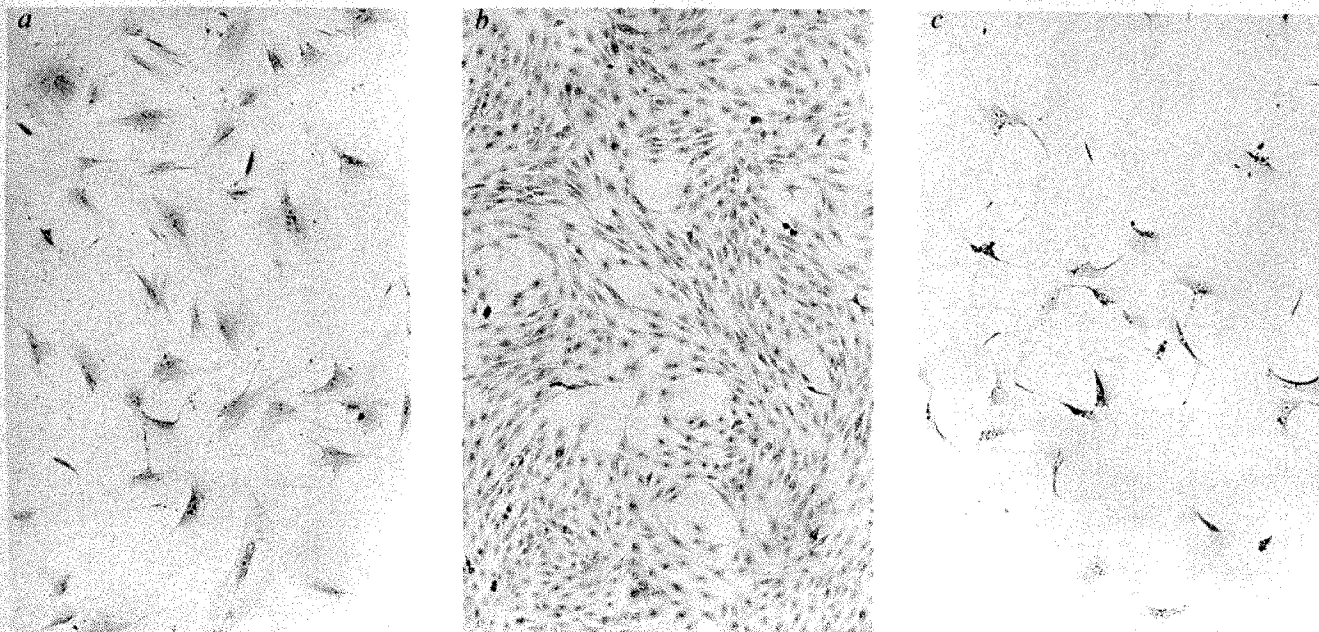
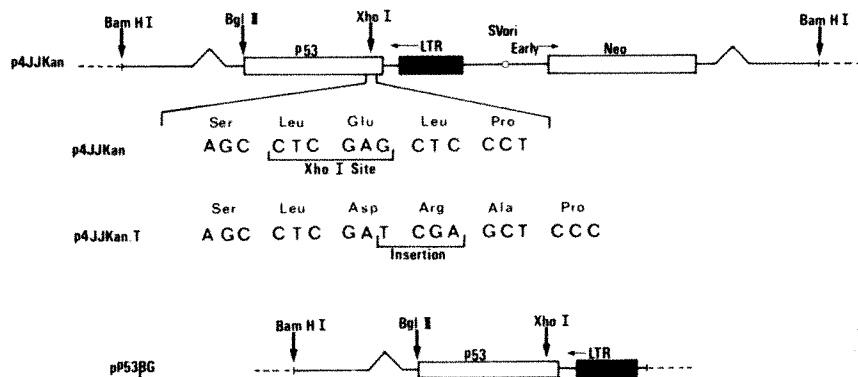


Fig. 1 Adult rat chondrocytes (WAXI). *a*, Calcium phosphate treated; *b*, after transfection with p4JJKan; *c*, after transfection with p4JJKan-T. **Methods:** WAXI cells were derived from the xiphisternum of adult Wistar rats. Fragments of xiphisternum were disaggregated with collagenase and the resulting cell suspension cultured in DMEM containing 10% FCS. When confluent, these cells were passaged using 0.1% trypsin, and low passage stocks were stored in liquid nitrogen. WAXI cells at passage 5 were established in dishes at 2×10^5 per dish in DMEM plus 10% FCS. The following day the cells were subjected to transfection using the standard calcium phosphate method¹⁹, with overnight incubation. Control cultures were treated with calcium phosphate only. Subsequently the cultures were fed thrice weekly and after 7 days were subcultured using a 1:5 split ratio into fresh dishes. Following this passage, untreated cultures regularly senesced. After 21 days culture (during which time cultures transfected with an active p53 expression vector (p4JJKan) had grown rapidly), all the cells were fed with medium containing G418 at $400 \mu\text{g ml}^{-1}$. After 21 days G418 treatment, all control untransfected cultures were dead.

Fig. 2 Schematic diagram of expression constructs containing murine p53 sequences. p4JJKan contains the sequences from pSV2neo (ref. 20) which confer G418 resistance on mammalian cells. Transcription of this gene in eukaryotic cells is directed by the SV40 early promoter. Transcription of pP53-5-derived murine p53 cDNA sequences¹⁸ is directed by the Rous Sarcoma Virus long terminal repeat (RSV LTR)^{21,22}. Splicing and polyadenylation signals for this transcript are provided by a second copy of the appropriate sequences from pSV2neo. p4JJKan directs the efficient synthesis of murine p53 protein in transient expression assays using monkey cos-1 cells and mouse specific anti-p53 monoclonal antibodies (data not shown). p4JJKan-T is essentially the same as p4JJKan, but the unique *Xho*I site in the p53 coding sequence has been cut, filled in with T4 polymerase and re-ligated. This generated a frameshift mutation. p4JJKan-T is unable to direct the synthesis of full-length murine p53 protein. This was confirmed in transient expression assays as above. p4JJKan and p4JJKan-T confer kanamycin resistance on their *Escherichia coli* hosts. Transcription of the p53 sequences in pP53BG is directed by the RSV LTR and splicing signals for this transcript are provided by a *Bal*I-*Bam*HI fragment of the mouse β -major globin genomic gene, which contains the second intron²³. A polyadenylation signal is provided by the 166 base pair *Hpa*I-*Bam*HI fragment of SV40 (nucleotides 2,666-2,533 on the SV40 genome). pP53BG carries the RTEM-1 β -lactamase gene of pBR322. Open boxes, coding sequence; solid boxes, LTRs; $\neg$, introns; $\cdots$, flanking pAT153 plasmid sequences.



ments, the constructs were transfected and the cultures were left for 7 days before passaging. They were then split 1:5 and passaged when necessary. On day 21 after transfection, the cultures were treated with the antibiotic G418. By this time, control WAXI cells had undergone senescence and were not proliferating. Cells transfected with the active p53 expression vector p4JJKan continued to proliferate and colonies grew up in $400 \mu\text{g ml}^{-1}$ G418 (see Fig. 1). Cells transfected with p4JJKan-T, which cannot direct the synthesis of full-length p53, provided occasional abortive colonies of senescing cells following G418 selection. Thus, p4JJKan-T conferred G418 resistance on the WAXI cells but failed to extend their lifespan. Immobilization of WAXI cultures by p4JJKan was reproducibly demonstrated in three separate experiments, and was associated with G418 resistance. We also transfected WAXI cells with the

p4JJKan vector and carried out the selection with G418 30 h later. By day 18 after the onset of G418 selection, colonies of proliferating cells were visible in p4JJKan-transfected dishes whereas p4JJKan-T-transfected dishes showed only occasional non-dividing cells (Table 1). We also co-transfected WAXI cells with pP53BG (Fig. 2) and pSV2neo, followed by G418 selection after 30 h. The results (Table 1) show that plasmid pP53BG, which uses mouse β -globin splice signals, also gives rise to colonies of proliferating cells by day 18, but pSV2neo does not. Lines of WAXI cells immortalized by p4JJKan and resistant to G418 have been maintained *in vitro* for over 200 doublings. They show no morphological evidence of senescence and continue to double every 18-20 h. Such cell lines are non-tumorigenic when injected into syngeneic or nude rats (5×10^6 cells per site).

Table 1 Phenotypes of rat chondrocytes after transfection with p53 expression vectors and/or T24 Ha-ras1

Plasmid	No. treated	G418-resistant colonies per dish	
Expt a			
None (calcium phosphate)	0/6	0, 0, 0	
p4JJKan	4/4	16, 18, 14	
p4JJKan · T	0/2	0, 0, 0	
pSV2neo	0/4	0, 0, 0*	
Foci per μg DNA per 10^6 cells			
Expt b			
p4JJKan		10, 9	
pP53 β G + psV-2-neo		15, 16	
pSV-2-neo		0, 0	
	Anchorage-independent growth (% colony formation)	Doubling time in medium plus FCS at:	
		10%	1%
Expt c			
None (calcium phosphate)	<0.01	>60 h	>60 h
p4JJKan	<0.01	18 h	>60 h
p4JJKan, then pT24-Ha-ras1	27, 32, 35	16 h	16 h
pT24-Ha-ras1	<0.01	ND	ND

Expt a, Rescue of WAXI cells from senescence by transfection with p53 expression vectors. G418 selection was carried out 21 days after transfection. 60-mm dishes were seeded with 5×10^4 cells and cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum (FCS). G418 (Geneticin) was used at $400 \mu\text{g ml}^{-1}$. Progressively growing colonies, resistant to G418, were seen only in dishes of WAXI cells previously transfected with the p4JJKan construct. Cells obtained from these colonies have continued to proliferate (>200 doublings) without showing morphological or proliferative evidence of senescence. **Expt b**, immortalization of WAXI cells by p53 expression vectors detected as resistant foci after treatment with G418 30 h post-transfection. Subconfluent 9-cm dishes of WAXI cells were transfected with plasmid DNA at $10 \mu\text{g}$ per dish and 30 h later were transferred to medium containing $400 \mu\text{g ml}^{-1}$ G418. Foci of rapidly proliferating cells were counted after 14 days. In dishes treated with the pSV-2-neo clone only, no foci were seen, and only occasional isolated senescent cells. **Expt c**, Anchorage and serum dependence of WAXI cells after transfection with p53 vectors and/or T24 Ha-ras1. The cell lines were assayed for anchorage-independent growth by suspension in 1.2% methylcellulose (Dow A4M) in DMEM plus 10% FCS cultured in 60-mm dishes over a base layer comprising 0.6% agarose in complete medium. The number of colonies present at 14 days was counted and expressed as a percentage of the total number of cells plated. ND, not determined.

* Occasional dishes show abortive colonies of senescent cells (see Fig. 1).

WAXI cells transfected with p4JJKan showed no evidence of crisis. Clones resistant to G418 were carried through 12 passages at a 1:10 split ratio and were examined for cloning efficiency in monolayer cultures. These cells had a high plating efficiency (65–70%) and furthermore, all individual colonies were morphologically identical. There was no evidence for the generation of senescent or terminally differentiated cells at any time after the initial outgrowth following transfection. Cloned immortalized WAXI cells maintained in complete medium showed a reproducible doubling time of 18 h. Our vector p4JJKan has also been examined for immortalizing activity in Syrian hamster dermal fibroblasts (R. Newbold, personal communication) with negative results. Interestingly, Newbold is also unable to induce immortalization of these cells with *myc* clones.

WAXI cells and the lines immortalized by the p53 constructs were transfected with cloned activated c-Ha-ras using pT24-Ha-ras-1, which contains a 6.6-kilobase *Bam*HI fragment of c-Ha-ras from the bladder cancer cell line T24, and were scored for transformed foci at day 21. Characteristic *ras* foci were seen only in the p4JJKan immortalized WAXI cells (Fig. 3); mortal

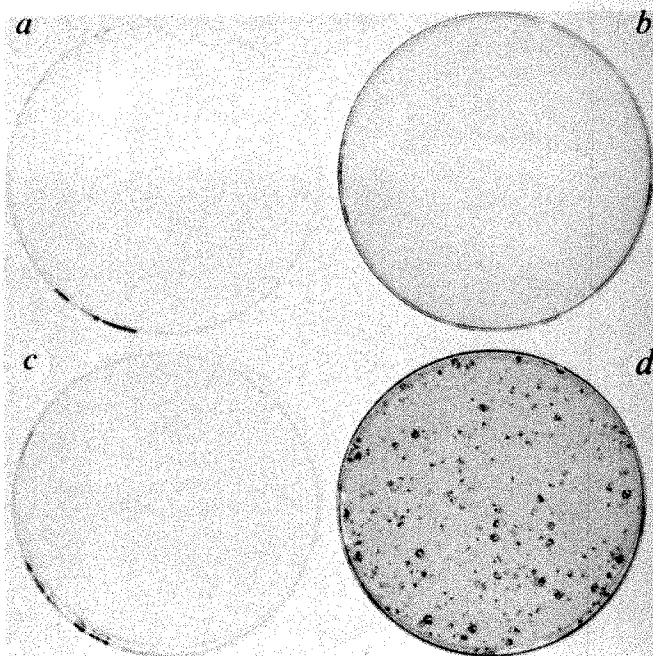


Fig. 3 Transforming activity of T24 Ha-ras1 on WAXI cells before and after immortalization by the p53 expression vector p4JJKan. *a*, WAXI cells; *b*, WAXI cells immortalized by p4JJKan; *c*, WAXI cells transfected with T24 Ha-ras1; *d*, p4JJKan-immortalized WAXI cells transfected with T24 Ha-ras1.

Methods: Monolayers of WAXI cells at passage 5, and p4JJKan-immortalized WAXI cells, established in Petri dishes at 2×10^5 cells per dish. The following day they were transfected with cloned T24 Ha-ras1, using the calcium phosphate method. $1 \mu\text{g}$ per 10^6 cells was used in each dish. After 21 days culture, characteristic *ras*-transformed foci were detected in the p4JJKan-immortalized WAXI cells only. Mortal WAXI cells showed no evidence of *ras* focus formation at any dose of T24 Ha-ras1 added (from 0.01 to $10 \mu\text{g}$ per dish).

WAXI cells were unaffected by the *ras* DNA. *ras*-transformed p53-immortalized WAXI cells were cloned and examined further. These cells show high anchorage-independent growth and rapid growth in low serum (Table 1). Furthermore, they induce tumours in nude rats. Immortal but untransformed WAXI cells are anchorage dependent and only grow in medium containing relatively high serum concentrations. Therefore, the major detectable phenotypic effect of the p53 expression construct p4JJKan is extension of cellular lifespan and it has no detectable effect on other phenotypes known to be associated with transformation. The observation that elevated levels of p53 are widely associated with malignant transformation induced by a variety of stimuli raises the possibility that this protein has a fundamental role in multi-stage carcinogenesis.

We thank Phil Mason for providing a mouse β -globin clone, Cori Gorman for a plasmid containing the RSV LTR, Christopher Dean for supplies of WAXI cells and Jean Marr for typing this manuscript.

Received 7 September; accepted 22 October 1984.

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Gene structure of human cardiac hormone precursor, pronatriodilatin

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Atrial cardiocytes contain granules typical of protein-secreting cells¹, and atrial extracts are known to contain a powerful natriuretic and diuretic activity² and to possess smooth muscle relaxant activity³. A variety of active atrial peptides have been isolated, including a family of related peptides showing natriuretic, diuretic and smooth muscle relaxant activities in rat⁴⁻⁹ and human atria¹⁰; these peptides were named atrial natriuretic factor (ANF). Another unrelated peptide from pig atria¹¹, cardiodilatin, is thought to possess only smooth muscle relaxant activity. Its partial amino acid sequence shows no homology with ANF sequences. The sequence analysis of a large form (106 amino acids) of ANF¹² and of ANF complementary DNA clones¹³⁻¹⁶ indicates that cardiodilatin and ANF peptides are synthesized from a common precursor. This precursor also contains a signal peptide sequence expected of a secretory protein¹⁷. We now describe the complete structure and sequence of the human gene for this novel hormone precursor that we call pronatriodilatin¹².

A rat pronatriodilatin cDNA clone¹³ was used as probe to isolate the human pronatriodilatin (hPND) gene from a human genomic DNA library¹⁸. A recombinant phage (λ hPND13) containing approximately 14 kilobases (kb) of human DNA was isolated. Restriction endonuclease cleavage sites were mapped in the human DNA by double digestions and fragments containing exon sequences were identified by hybridization with the rat pronatriodilatin cDNA probe. As the rat cDNA clone might be less homologous to human sequences in the untranslated regions, we localized exons containing these sequences with radioactive cDNA probes complementary to human poly(A)⁺ atrial RNA. A first probe made by priming cDNA synthesis with oligo-d(T)₁₂₋₁₈ was used to map the exon containing 3'-untranslated sequences. Hybridization of hPND gene restriction fragments with this probe also suggested the presence of a transcribed and possibly repeated sequence element in the large intron of the gene flanking the *Ava*I site. This was confirmed by hybridization of the same restriction fragments with nick-translated total human DNA. DNA sequence determination (see below) indicates that this region contains repeated sequences of the *Alu* family¹⁹. The first exon of the gene was localized using a second human cDNA probe made by priming with a synthetic eicosamer complementary to the previously determined sequence encoding amino acids 31-37 of the precursor.

The complete structural organization of the hPND gene is shown in Fig. 1. The 2.6-kb *Bam*HI-*Eco*RI fragment (Fig. 1b) contained all the hybridizing sequences and was subcloned in pBR327 for further characterization. DNA sequencing was performed by the chemical²⁰ and chain termination²¹ methods. For the latter, single-stranded templates were obtained by subcloning in M13mp8 and mp9 (ref. 22) (Fig. 1d). The hPND gene sequence, including about 500 base pairs (bp) upstream of the

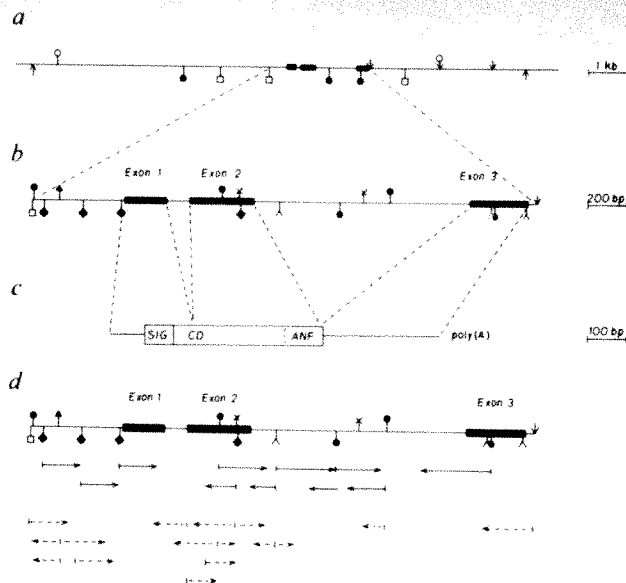


Fig. 1 a, Structure of human pronatriodilatin (hPND) gene. A map of restriction enzyme cleavage sites was obtained by double digestion of a bacteriophage λ Charon 4A recombinant containing ~14 kb of human DNA. Exon sequences (bars) were localized on this map by hybridization with various rat and human probes as outlined in the text. The following symbols are used for the restriction enzyme cleavage sites: *Eco*RI($\downarrow$), *Hind*III($\uparrow$), *Xba*I(∇), *Bam*HI($\square$), *Pst*I($\bullet$), *Ava*I($\circ$), *Kpn*I($\blacktriangle$), *Xho*II(∇), *Pvu*II($\blacklozenge$) and *Nco*I($\wedge$). b, Detailed restriction map of the 2.6-kb *Bam*HI-*Eco*RI fragment containing all the sequences hybridizing with pronatriodilatin cDNA probes. A subclone containing this fragment in pBR327 was used for sequencing. c, Schematic diagram of hPND mRNA showing regions present in each of the three exons. The translated portion of the mRNA is shown as an open box which includes the putative signal peptide sequence (SIG), the sequence homologous to pig cardiodilatin (CD) and the ANF sequence. The ANF sequence is preceded by a broken vertical line to indicate the putative nature of this processing site because many ANF peptides with variable-length amino termini have been described⁴⁻⁹. d, Strategy used for DNA sequence determination of hPND gene. Arrows below the diagram of the 2.6-kb *Bam*HI-*Eco*RI fragment indicate the length and direction of sequences obtained by the chain termination²¹ (solid arrows) or chemical²⁰ (broken arrows) methods. Single-stranded templates were obtained by subcloning in M13mp8 and mp9 (ref. 22) fragments of the 2.6 kb *Bam*HI-*Eco*RI produced with *Ava*I, *Ava*I + *Nco*I, *Pvu*II, *Pst*I or *Xho*II. Fragments were labelled at their 3' ends by filling-in²⁵ with the Klenow fragment of *Escherichia coli* DNA polymerase I for chemical sequence determination. The sites used for labelling were: *Ava*I, *Ava*II, *Bam*HI, *Eco*RI, *Fok*I, *Hin*fI, *Nco*I, *Sau*I.

Methods: A human genomic DNA library¹⁸ (kindly provided by Dr Tom Maniatis) was screened by filter hybridization²⁶ with a nick-translated²⁷ rat pronatriodilatin cDNA probe¹³. Hybridizations were performed at 55 °C in 20% formamide, 6 $\times$ SSC (20 $\times$ SSC is 3 M NaCl, 0.3 M sodium citrate), 0.05 M sodium phosphate buffer pH 6.8, 5 $\times$ Denhart's solution²⁸, 50 μ g ml⁻¹ sonicated denatured salmon sperm DNA. After hybridization overnight, the filters were washed at 50 °C in 1 $\times$ SSC and 0.1% SDS. Exon sequences were localized in the recombinant λ hPND13 by hybridization with the rat cDNA probe in the same conditions as above. The localization of 5' and 3'-untranslated sequences was ascertained with homologous human cDNA probes. These probes were synthesized in reactions containing 80-200 μ g ml⁻¹ human atrial poly(A)⁺ RNA, 400 μ g ml⁻¹ oligo-d(T)₁₂₋₁₈ or 6-7 μ g ml⁻¹ synthetic 20-mer (5'-AGGTCTGCGTTGGACACGGC-3') as primers, 200 U ml⁻¹ avian myeloblastosis virus reverse transcriptase and buffer (50 mM Tris-HCl pH 8.3, 60 mM KCl, 8 mM MgCl₂, 5 mM dithiothreitol).

transcription initiation site, is shown in Fig. 3 along with the translation product.

From a comparison of human¹⁶ and rat¹³⁻¹⁵ cDNA sequences, we conclude that the hPND gene is constituted of three exons. The first exon contains 5'-untranslated sequences, the initiator

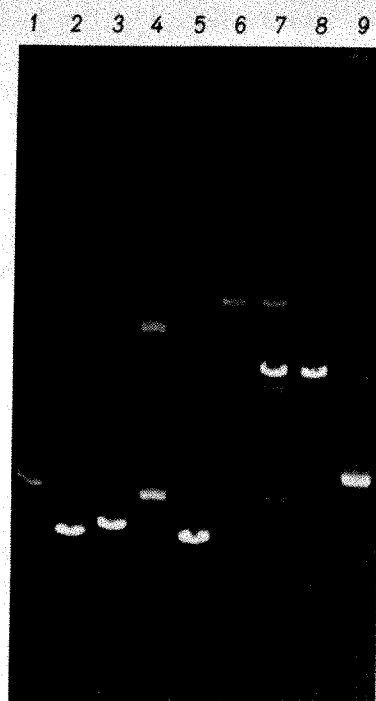


Fig. 4 Allelic variation in two hPND cDNA clones. The two hPND cDNAs were inserted in the *Pst*I site of pBR322 using dG:dC homopolymer tailing. Both cDNAs are about 350 bp, start within ANF-coding sequences and extend to the 3' end of hPND mRNA. In both cases, 200 bp were sequenced from the 5'-end to the *Pst*I site (underlined in Fig. 3) in the 3' non-coding region; only two nucleotide differences were found between the two cDNA sequences. One cDNA is identical to the genomic sequence (Fig. 3) and to the published hPND cDNA sequence¹⁶. One nucleotide difference in the stop codon of the variant cDNA makes it similar to the rat cDNA sequence¹³ and introduces a new *Kpn*I site in the cDNA as confirmed by the restriction enzymes digestions shown here. The two cDNA clones were characterized by electrophoresis on 0.8% agarose gel: lanes 1-3, variant cDNA; lanes 4-7, wild-type cDNA. Lanes 1, 4, supercoiled plasmids; lanes 2, 5, digestion with *Nco*I which cuts both cDNA inserts twice; lanes 3, 6, digestion with *Kpn*I; lane 8, same as lane 7 but including another plasmid linearized by *Kpn*I as internal control. This control plasmid is shown linearized by *Kpn*I in lane 8 and in supercoiled form in lane 9.

two arginine residues after the ANF sequence. In addition to hPND cDNA clones that are identical to the cloned genomic sequence, we have isolated a short cDNA clone (~350 bp) that, like the rat cDNA clones, encodes two arginine residues after the ANF sequence¹³. This cDNA clone differs at only two positions (within the 200 bp sequenced) from the other human sequences. The first nucleotide difference changes the stop codon into an arginine triplet and creates a new *Kpn*I restriction site in the cDNA clone (Fig. 4). The other nucleotide change is in the 3'-untranslated region; the two nucleotides that differ from the genomic sequence are shown on the complete sequence (Fig. 3). As Southern blot experiments (ref. 16 and our work, not shown) suggest the presence of only one hPND gene, these two clones are likely to represent different alleles of the hPND gene.

We thank Dr Jacques Genest for his support and the surgical team of the Institut de cardiologie de Montréal for the human atrial biopsy material used in cDNA cloning. We also thank Céline Forget for oligonucleotide synthesis, Mamdouh Mikhail for his help with the sequence analysis programs and Diane Lapointe for typing the manuscript. J.D. is supported by grants from the MRC and the National Cancer Institute of Canada.

Received 24 July; accepted 18 October 1984.

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Nucleotide sequence of the gene encoding human atrial natriuretic factor precursor

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The mammalian atrium is an endocrine organ that may be involved in the control of blood pressure and extracellular fluid volume. A series of peptides¹⁻⁶, which seem to be associated with atrium-specific secretory granules, have potent natriuretic, diuretic and smooth muscle relaxant activities. Sequence determination of several of these peptides, which range from 21 to 126 amino acids long, shows that they form a family, derived from a common precursor⁶⁻¹². Rat and human complementary DNAs that encode the precursor to the various peptides, collectively called atrial natriuretic factors (ANFs), have been cloned¹³⁻¹⁷. Nucleotide sequencing showed that the ANFs are located at the C-terminus of a polypeptide of relative molecular mass 13,000. We describe here the isolation and characterization of the corresponding human gene. Two introns interrupt the gene; one is located in the region coding for the N-terminus of the precursor and the other separates the codon for the C-terminal tyrosine from the rest of the peptide.

Using a rat ANF cDNA¹³ as probe, we isolated a phage containing a 16.6-kilobase (kb) human genomic fragment. We have subcloned its complete hybridizing region, present on a 2.6-kb *Eco*RI-*Bam*HI fragment, and determined its nucleotide sequence (Fig. 1). The first exon contains the 5'-untranslated region, the signal peptide and the 16 amino acids following the signal peptidase cleavage site. The second exon encodes the rest of the propeptide except for the C-terminal tyrosine, which is found at the 5' end of the third exon. The sequences at each of the exon-intron junctions match the consensus splice site sequence¹⁸. A TATA box occurs at position 442 and two potential CCAAT boxes occur at positions 235 and 342 (ref. 19).

The second intron contains the sequence GCCATCTATT-GTCCT (positions 1,238-1,297), a potential binding site for the glucocorticoid hormone-receptor complex. Karin *et al.*²⁰ described the consensus binding site sequence $\uparrow$ GGT $\uparrow$ CA $\uparrow$ CTGT $\uparrow$ CT. Although the first four bases of the consensus do not match the putative binding site in the human gene, 10 of the last 11 do. This includes the hexanucleotide

Human	ATG AGC TCC TTC TCC ACC ACC ACC GTG AGC TTC CTC CTT TTA CTG GCA TTC CAG CTC CTA GGT CAG ACC AGA GCT	AAT CCC
Rat	G	T --- AA G T C C C T TG C C T T G A
Human	met ser ser phe ser thr thr thr val ser phe leu leu leu leu ala phe gin leu leu gly gin thr arg ala	asn pro
Rat	gly	ile --- lys gly phe phe -10 trp pro his ile gly -1 +1
Human	ATG TAC AAT GCC GTG TCC AAC GCA GAC CTG ATG GAT TTC AAG	-----122 bp----- AAT TTG CTG GAC CAT TTG GAA GAA AAG
Rat	G A G G A T	C C A C C G G
Human	met tyr asn ala val ser asn ala asp leu met asp phe lys	asn leu leu asp his leu glu glu lys
Rat	val ser thr	
	+10	+20
Human	ATG CCT TTA GAA GAT GAG GTC GTG CCC CCA CAA GTG CTC AGT GAG CCG AAT GAA GAA GCG GGG GCT GCT CTC AGC CCC CTC	
Rat	G G A T G G CC G C A CC T	G A T T
Human	met pro leu glu asp glu val val pro pro gin val leu ser glu pro asn glu glu ala gly ala leu ser pro leu	
Rat	val met ala gin thr asp	ser
	+30	+40 +50
Human	CCT GAG GTG CCT CCC TGG ACC GCG GAA GTC AGC CCA GCG CAG AGA GAT GGA GGT GCC CTC GGG CGG GGC CCC TGG GAC TCC	
Rat	T T T A G T T T	T C C
Human	pro glu val pro pro trp thr gly glu val ser pro ala gin arg asp gly gly ala leu	gly arg gly pro trp asp ser pro
Rat	ser	
	+60	+70
Human	TCT GAT CGA TCT GCC CTC CTA AAA AGC AAG CTG AGG GCG CTG CTC ACT GCC CCT GCG AGC CTG CGG AGA TCC AGC TGC TTC	
Rat	C A T G A	G G A G A
Human	ser asp arg ser ala leu leu lys ser lys leu arg ala	leu leu thr ala pro arg ser leu arg arg er ser cys phe
Rat		ala gly
	+80	+90 +100
Human	GGG GCG AGG ATG GAC AGC ATT GGA GCC CAG AGC GGA CTG GGC TGT AAC AGC TTC CGG	-----1093 bp----- TAC TGA AGA TAA
Rat	T T	C
Human	gly gly arg met asp arg ile gly ala gin ser gly leu gly cys asn ser phe arg	tyr stop
Rat	ile	arg arg stop
	+110	+120 +126

Fig. 2 Comparison of the human and rat sequences. The amino acid sequences of the human and rat ANF precursor are aligned with their corresponding nucleotide sequences. The rat sequences are shown only where they differ from the human sequences. Numbering refers to the human amino acid sequence, with +1 referring to the N-terminal Asn of the propeptide. Alignment of the rat and human sequences is perfect except at codon -19, which is not present in the rat precursor. Potential proteolytic processing sites^{10,12-17} are boxed. Rat sequences are from refs 13, 14 and 16.

sequence, TGT²⁰CT, found in all glucocorticoid receptor binding sites^{20,21}. As with the human growth hormone gene, this site occurs within an intron²². Also within the second intron are two tandem *Alu* repeats, which are 76% homologous, spanning bases 1,356-1,646 and 1,647-1,942. Each shows a marked homology (70-86%) with the various *Alu* repeat sequences described by Deininger *et al.*²³.

Differences between the rat and human propeptide amino acid sequences (+1 to +126, Fig. 2) occur at 17 positions, giving an 86.5% amino acid sequence homology. The nucleotide sequence homology over the same region is 85.4%. The homology is much lower between the signal peptide sequences of the two species, as has also been observed in inter-specific comparisons of somatostatin and proopiomelanocortin genes²⁴⁻²⁶. In each case, however, the hydrophobic character of the signal peptide is preserved. Also, the human gene contains a single polyadenylation signal, AATAAA (ref. 27), whereas the rat cDNA has two^{13,14,16}.

Active peptide hormones are not usually interrupted by introns, in agreement with the suggestion that functional domains are defined by exons²⁸. The intron that occurs within the ANF coding sequence, however, separates the C-terminal tyrosine from the rest of the sequence. Note, in this context, that *in vitro* assays comparing the natriuretic and vasoactive properties of synthetic des-Tyr-ANF with native ANF have shown that the C-terminal tyrosine may not be required for biological activity¹. Comparison of the rat cDNA with the human gene structure suggests that the ANF gene may have evolved from a longer, possibly multi-functional gene. The C-terminal Arg-Arg in the rat may be a remnant of the original structure, and the single amino acid encoded in the third human exon may represent a further evolutionary step towards a two-exon gene.

The physiological role of the atrial natriuretic peptides is unclear. Several schemes by which the ANF precursor is processed to give short biologically active peptides have been proposed^{10,12-17}, but whether any of these occurs *in vivo* remains to be determined. Evidence has been recently obtained that ANF peptides circulate in dog plasma and that their levels correspond to variations in blood pressure (J.A.L., unpublished

results). Knowledge of the gene sequence for the human ANF precursor should now allow us to investigate the biological activities of various encoded peptides and to study their role in controlling human blood pressure and fluid volume.

We thank M. Brewer for technical assistance, T. Maniatis and P. Frossard for gifts of the human genomic library and human genomic DNA, J. Iwasa for oligonucleotide synthesis, our colleagues at CBI for their encouragement and for critically reading the manuscript, and E. Pond for typing the manuscript. This work was supported by Biotechnology Research Partners Ltd.

Received 16 July; accepted 18 September 1984.

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Identification of the coding sequence for a reverse transcriptase-like enzyme in a transposable genetic element in *Drosophila melanogaster*

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The largest group of transposable elements in *Drosophila melanogaster*, *copia*-like elements^{1,2}, share some important structural features with and are intimately related in evolution to vertebrate retroviruses²⁻¹². To further clarify the relationship between retroviruses and *copia*-like transposable elements, we set out to determine the complete nucleotide sequence of the genome of 17.6, which has long terminal repeats homologous in nucleotide sequence to those of avian leukaemia-sarcoma virus¹². We report here that 17.6 contains three long open reading frames comparable with *gag*, *pol* and *env* genes in retrovirus. At the level of amino acid sequence, the longest open reading frame of 17.6 includes a coding sequence similar to that for reverse transcriptase, suggesting a role for this enzyme in the life cycle of some *Drosophila copia*-like elements, analogous to the situation in retrovirus.

The nucleotide sequence of the internal body (the region flanked by long terminal repeats (LTRs)) of 17.6, inserted in histone genes in λ hist 17.6^{12,13}, was determined by the Maxam and Gilbert procedure¹⁴. The resultant nucleotide sequence was combined with the LTR sequences previously published¹² to give the nucleotide sequence of the 17.6 genome (7,439 base pairs (bp)): translation of the nucleotide sequence showed 17.6 to contain three long open reading frames (ORFs), 1, 2 and 3 (Fig. 1). Other ORFs (locations not shown here) are too short to encode the polypeptides of more than 150 amino acids. The majority of 17.6 elements distributed on the chromosome may contain the nucleotide sequences for these three long ORFs, as the restriction maps of more than half of the 17.6s isolated from 18 chromosomal loci were identical to the 17.6 map examined here¹⁵. In three 17.6s examined, including the one discussed here, no differences were found in the nucleotide sequence of a 300 bp region arbitrarily chosen within the internal body.

The ORFs 1, 2 and 3 of 17.6 show similarities in size and location to *gag*, *pol* and *env* genes, respectively, of avian leukaemia virus (ALV)¹⁷ and Moloney murine leukaemia virus (MoMuLV)¹⁶ (Fig. 3). Furthermore, as with the retrovirus proviruses, all these long ORFs of 17.6 were found to reside on a specific DNA strand simultaneously containing the nucleotide sequences corresponding to a Hogness box, polyadenylation signal, primer binding site and polypurine sequence in ALV¹² (see Fig. 1). A 540-bp noncoding sequence, very rich in A+T content, was also located upstream from the ORF 1 (Fig. 1). As shown below, the ORF 2 of 17.6 includes the coding sequence for the enzyme similar to reverse transcriptase, which, in the case of the retrovirus, is coded by *pol*.

Virus-like particles (VLPs) with *copia* RNA and the reverse transcriptase-like enzyme activity have been identified in *D. melanogaster* and *copia* RNA encapsidated is considered to have a weak mRNA activity for making the polypeptides immunologically related to the VLP coat proteins¹¹. However, it is not clear whether the *copia* genome contains the gene for the reverse transcriptase-like enzyme. This fact, along with the above finding that 17.6 is similar in genome organization to retroviruses, prompted us to survey the coding sequence for the

enzyme similar to reverse transcriptase of retrovirus within the 17.6 genome. We compared the amino acid sequences of the putative polypeptides expected to derive from the three ORFs of 17.6 with those of the reverse transcriptases of MoMuLV¹⁶ and Rous sarcoma virus (RSV)¹⁷, and that of the putative reverse transcriptase of cauliflower mosaic virus (CaMV)¹⁹. The ORF 2 protein of 17.6 was found to contain a 200–300 amino acid long domain which could match some portions of the *pol* gene products of MoMuLV and RSV, and of the counterpart of CaMV (Fig. 2). The amino acid sequence homology between 17.6 and MoMuLV was estimated to be 25%, whereas those between 17.6 and RSV and between 17.6 and CaMV were 20 and 30%, respectively. We will hereafter refer to the regions with these homologous amino acid sequences and their DNA counterparts as RT polypeptides and RT DNAs, respectively.

It is noteworthy that the RT polypeptides of MoMuLV and RSV correspond to the regions containing the amino acid sequences which have been suggested as being conservative among various reverse transcriptases and similar enzymes, and in charge of the polymerase activity²⁷ (Fig. 3). Furthermore, the RT polypeptide of 17.6 was found to contain 9 of the 10 amino acids that are invariant in all reverse transcriptases so far examined²⁷ (Fig. 2). In addition, the relative positions of the RT DNAs of 17.6, MoMuLV and ALV (RSV), were found to be similar to one another, assuming the ORF 2 of 17.6 to be identical to the *pol* genes of the retroviruses (Fig. 3). Thus, it is probable that the RT DNA of 17.6 is the major coding sequence for the enzyme similar to the reverse transcriptase of retrovirus. In a separate experiment (unpublished), we determined the nucleotide sequences of the entire 297 (ref. 6) genome, and a portion of the 412 (ref. 3) genome and found that these elements also contain the nucleotide sequences corresponding to the RT DNA. Thus the nucleotide sequence of the RT DNA seems to be conserved among at least some classes of *Drosophila copia*-like element; the RT polypeptides have played an important role in maintaining these elements as transposable elements during evolution.

The carboxyl-terminal regions of the reverse transcriptases of ALV and MoMuLV contain DNase activity^{16,18,20}, probably required for integration²⁸. Interestingly, the ORF 2 proteins of 17.6 and 297 contain the regions with highly conservative amino acid sequences near their carboxyl ends (unpublished data). We have identified recently the significant amino acid sequence homology between these carboxyl terminal domains of ORF 2 proteins of 17.6 and 297, and the DNase regions of ALV and MoMuLV, suggesting that even the overall organization of these genes is similar to that of the *pol* genes of retroviruses (manuscript in preparation). ORF 1 of 17.6 and 297 may be related also to *gag* of MoMuLV, as weak homology at the level of amino acid sequence could be detected.

17.6 contains nucleotide sequences that apparently correspond to a primer binding site and polypurine sequence, both mandatory for the initiation of DNA synthesis in reverse transcription in a retrovirus system¹². As described above, we have confirmed that 17.6 has the coding sequence for reverse transcriptase-like enzyme. Furthermore, *Drosophila* appears to contain a candidate tRNA of 297 primer since our data (unpublished) have revealed that as with retrovirus primer tRNA^{16,17}, the 3'-terminal 18-bp fragment of one of the *Drosophila* serine tRNAs is complementary to the 18-bp, putative primer binding site of 297. Circular DNAs of 17.6 and 297 which appear to correspond to the circular retrovirus DNAs²¹ have also been identified in *Drosophila* (ref. 10 and our unpublished data). Thus, it is quite conceivable that the RNAs of 17.6 and other similar elements are reverse transcribable by a mechanism similar to that of retrovirus, and that the resultant DNAs or their derivatives are used as intermediates for integration. VLPs, which may be required for the effective reverse transcription, have been found only in the case of *copia*.

Our results support the view that *copia*-like transposable elements in *Drosophila* and retroviruses in vertebrates bear a very close evolutionary relationship, but do not reveal whether

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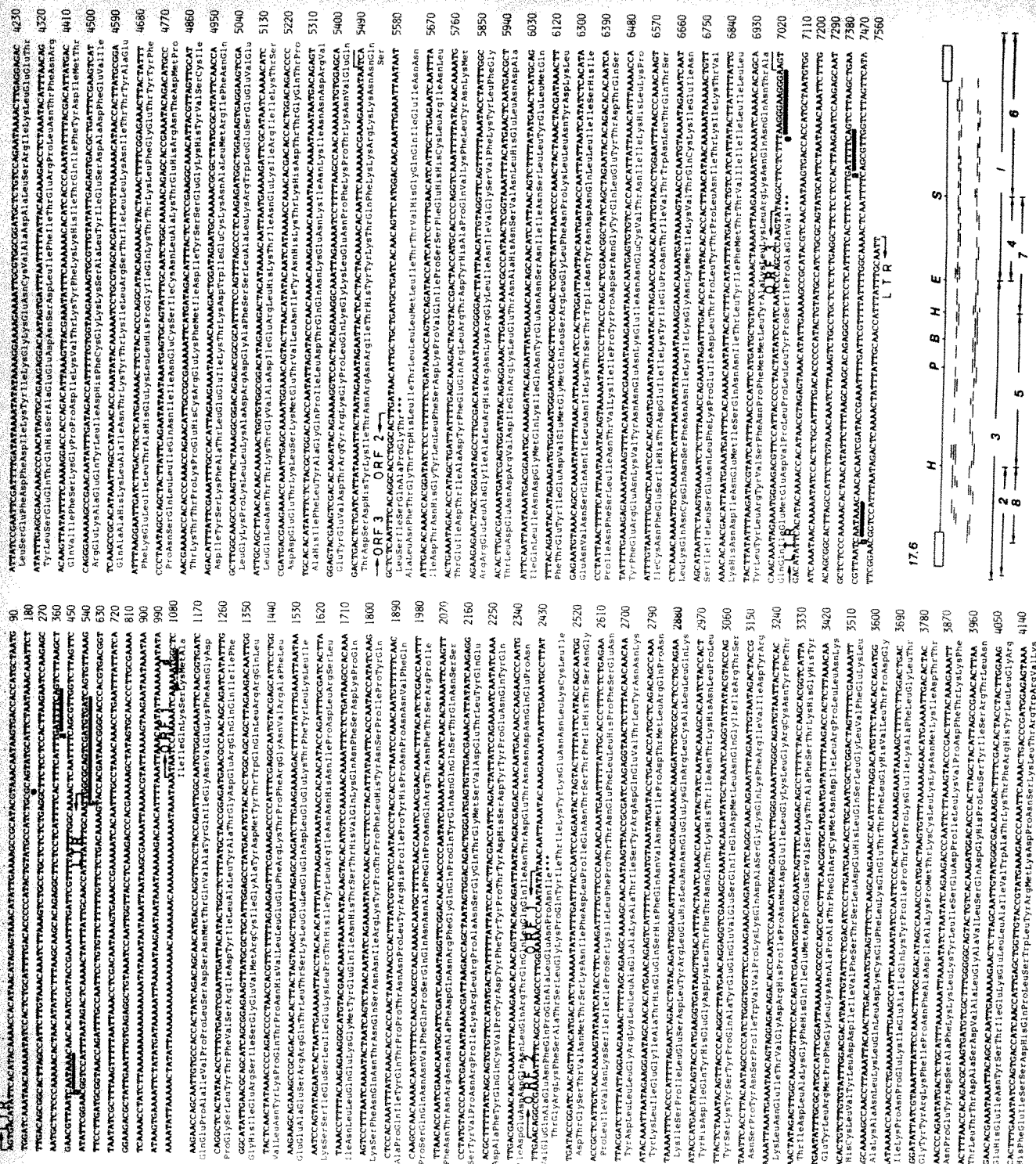


Fig. 1 The complete nucleotide sequence of the 17.6 genome, presented as the putative sense strand which includes three open reading frames. The ranges of three ORFs, which are translated below the nucleotide sequence, are shown by L-shaped arrows. *, The termination codon of each ORF; underlines, nucleotide sequences corresponding to a, the Hogness box, b, polyadenylation signal, c, primer binding site and e, polypurine sequence in ALV¹². The underline labelled d shows the initiation signal for translation²³ of ORF 1.

Methods: The DNA extracted from *A. hist* 17.6 (containing a histone gene repeat inserted by 17.6 element) was digested by various restriction enzymes and the resultant DNA segments containing 17.6 pieces were recloned using pUC9¹⁵ as a cloning vector. The sizes and locations of the cloned DNA segments are shown by thick lines with arrowheads and numbers in the scheme on the right. After *Escherichia coli* cells [JM83 (ref. 24) or GM48 (dcm-6, dam-3) (ref. 25) harbouring a desired plasmid] were cultured in 100 ml L broth for 15 h at 37 °C with shaking, plasmid DNA was extracted according to Clewell and Helinski²⁶ and purified by banding in a CsCl gradient containing 50 µg ml⁻¹ of ethidium bromide. Closed circular DNA was collected, treated with 5 M NaCl-saturated isopropanol several times, dialysed against 1 mM Tris-HCl (pH 7.4) + 0.1 mM EDTA and stored at 4 °C until use. Plasmid DNA was digested with a suitable restriction enzyme, end-labelled with either kination or filling-in, redigested with the second restriction enzyme and sized on agarose or polyacrylamide gels by electrophoresis. The desired DNA segment extracted was then subjected to the partial chemical degradation method of Maxam and Gilbert¹⁴. The overall strategy for sequencing is depicted by thin underlines in the lower margin illustration. About 90% of the final sequence was determined for both strands of duplex DNA to minimize artefacts such as compression; upper and lower lines show the regions of the DNA strands sequenced. B, E, H, P and S, the cutting sites of the major restriction enzymes used for cloning and sequencing, B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I.

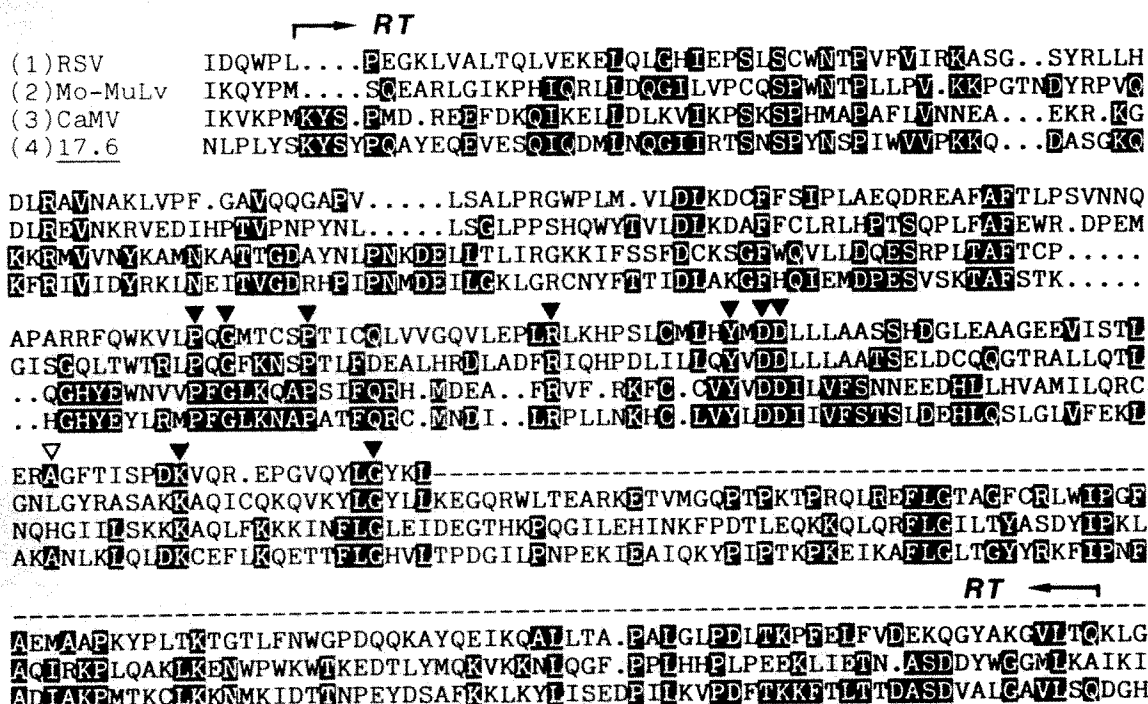


Fig. 2 Comparison of the amino acid sequences of RT polypeptides from various sources. The amino acid sequences were deduced from the nucleotide sequences (RSV, ref. 17; MoMLV, ref. 16; CaMV, refs 19, 27; 17.6, Fig. 1 (this work)). L-shaped arrowheads labelled RT, ranges of the RT polypeptides. Lines 1, 2, 3, 4; amino acid sequences of the RT polypeptides of RSV, MoMLV, CaMV and 17.6, respectively. White letters enclosed by filled boxes, homology to 17.6 in lines 1, 2 and 3, and homology to RSV, MoMLV or CaMV in line 4. Filled triangles, locations of 9 of the 10 invariant amino acids²⁷ found in 17.6; an open triangle, amino acid not found as an invariant in 17.6.

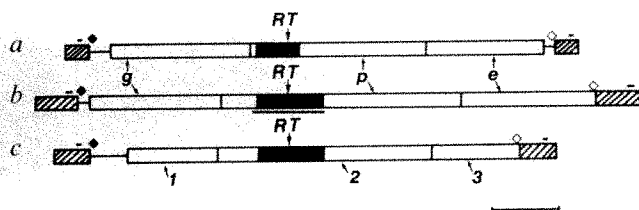


Fig. 3 Comparison of the genome structures between retrovirus proviruses and a copia-like element, 17.6. *a*, *b*, Genome organizations of the integrated proviral forms of ALV and MoMLV, deduced from the RNA genome structures^{16,17}, respectively. In particular, the structure of the ALV provirus is identical to that of RSV without *src* gene and one *DR*¹⁷. The structure of 17.6 (2) was deduced from the nucleotide sequence shown in Fig. 1. Hatched boxes, LTRs; filled boxes, RT DNAs (see Fig. 2); open boxes, ORFs of 17.6 and the genes of retroviruses. Filled lozenges, positions of the primer binding sites in *a* and *b* and the putative primer binding site in *c*; open lozenges, positions of polypurine sequences; small rectangles over the hatched LTR boxes, regions containing the Hogness box and polyadenylation signal or their related sequences^{12,16,17}. Scale bar, 1,000 bp. Arrows labelled RT, positions of the nucleotides corresponding to the most conservative amino acid sequences, YXDD, in the RT polypeptides (see Fig. 2). Underline in *b*, region of amino acid sequence highly homologous to that of the reverse transcriptase-like enzyme of CaMV (see ref. 27). *g*, *gag*; *p*, *pol*; *e*, *env*; 1, ORF 1; 2, ORF 2; 3, ORF 3.

the common ancestor is a retrovirus^{11,12} or a transposable element²².

This work was supported by grants (to K.S.) from the Ministry of Education, Science and Culture, Japan.

Received 31 July; accepted 2 October 1984.

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Nicotinic receptor stimulation activates enkephalin release and biosynthesis in adrenal chromaffin cells

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Neuroendocrine cells release a portion of their stored secretory hormone content when exposed to tissue-specific secretagogues. In the case of the adrenal medulla, catecholamines and enkephalin peptides, as well as other secretory proteins, are secreted in response to acetylcholine, which is released onto cholinergic recep-

tors on chromaffin cells upon splanchnic nerve stimulation *in vivo*¹⁻³. Secretagogue stimulation thus depletes intracellular stores of exportable hormone. We were interested to know whether the signal for exportable hormone release might also function as a signal for compensatory hormone repletion by enhancing the biosynthesis of the released hormone(s). Accordingly, we have investigated the effect of nicotinic receptor stimulation on Met-enkephalin peptide biosynthesis and expression of proenkephalin messenger RNA in primary cultures of bovine chromaffin cells. Our results, reported here, suggest a model for stimulus-secretion-synthesis coupling in which nicotinic receptor occupancy activates two pathways. One pathway, dependent on calcium and not mimicked by increased intracellular cyclic AMP, leads to exocytotic hormone release; the other, probably via a calcium-dependent increase in intracellular cyclic AMP, leads to a compensatory increase in intracellular enkephalin through activation of transcription of the proenkephalin structural gene.

Chromaffin cells were dissociated from bovine adrenal medulla and maintained in primary culture for 72-96 h⁴. Medium was then removed from the cells and replaced with fresh medium containing nicotinic agonists and/or antagonists. Exposure to 10 μ M nicotine for 24-72 h resulted in a gradual increase in total Met-enkephalin immunoreactivity in the cultures (Fig. 1). This increase was completely blocked in the presence of 5 μ g ml⁻¹ cycloheximide (data not shown). It was preceded by an increase in mRNA coding for proenkephalin (mRNA^{enk}) which is already maximal at 24 h, when peptide levels begin to rise (Fig. 1). Nicotinic induction of enkephalin biosynthesis was maximal at the dose used (5-10 μ M) and was not observed with doses of nicotine $\geq$ 1,000 μ M. This desensitization to nicotine is also characteristic of nicotine-induced enkephalin and catecholamine release^{2,4}. The induction of mRNA^{enk} by nicotine is quite rapid: an increase in mRNA^{enk} is detectable within 2 h of exposure to 5 μ M nicotine and is maximal by 8 h (Fig. 2). This rapid time course suggests that nicotine is acting to increase mRNA^{enk} by enhancing proenkephalin gene transcription rather than by inhibiting its degradation. Enhanced transcription of the proenkephalin gene is also suggested by a rapid increase in nuclear proenkephalin precursor RNA following exposure to nicotine (H.-U.A., unpublished observations). Nicotine first causes an actual depletion of intracellular Met-enkephalin, due to its release into the culture medium. By 25 h, however, intracellular peptide stores have returned to pre-stimulation levels, indicating that compensatory biosynthesis has occurred (Fig. 2).

Nicotinic stimulation of Met-enkephalin biosynthesis, enkephalin release and levels of mRNA^{enk} are all blocked by addition of hexamethonium, a nicotinic antagonist (Table 1). In addition, induction of mRNA^{enk} by 10 μ M nicotine is blocked by hexamethonium added as long as 1 h after nicotine, suggesting that in these experimental conditions nicotinic induction of enkephalin biosynthesis is not secondary to a feedback effect of secretory products (for example, enkephalin, ATP, catecholamines); these are maximally released from the cells during the first 15 min of exposure to nicotine (L.E.E., unpub-

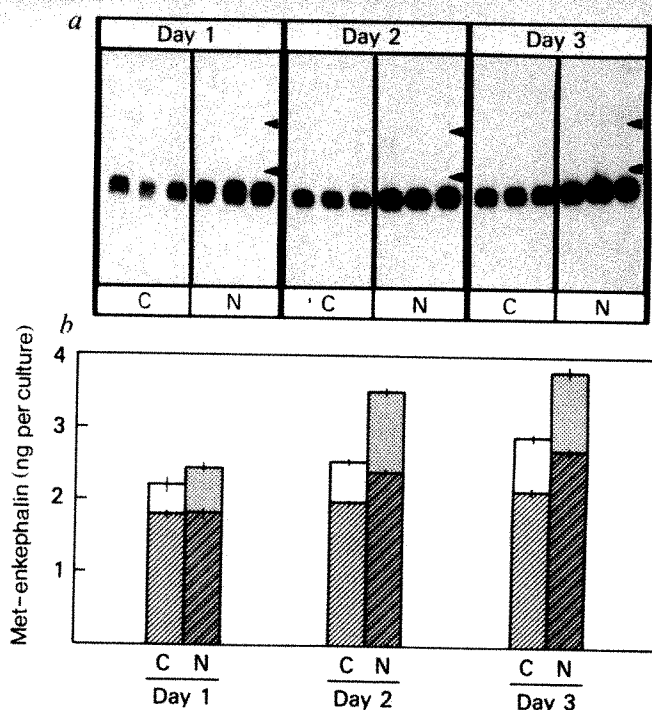


Fig. 1 Effect of chronic exposure to nicotine on mRNA^{enk} and immunoreactive Met-enkephalin in cultured chromaffin cells. *a*, Autoradiograms of Northern blots prepared from chromaffin cell total nucleic acids 1, 2 or 3 days following exposure to 5 μ M nicotine (N) or vehicle (C) and hybridized with a ³²P-labelled 400-base-pair *Pst*I restriction fragment of bovine proenkephalin A cDNA¹³ as described previously^{5,6}. Arrows indicate positions of 28S and 18S RNA. Autoradiograms were scanned densitometrically and corrected for total RNA applied in each sample by scanning densitometrically negative photographs of UV-transilluminated, ethidium-stained gels run in parallel to the gels used for Northern transfer. These values were (control at each time point corrected to 100%): day 1, C = 100 $\pm$ 5, N = 152 $\pm$ 10; day 2, C = 100 $\pm$ 6, N = 145 $\pm$ 8; day 3, C = 100 $\pm$ 9, N = 183 $\pm$ 22, $\bar{x} \pm$ s.e.m., $n = 3$. *b*, Immunoreactive Met-enkephalin was measured by radioimmunoassay using the RB-4 antiserum exactly as described in ref. 6. N, values measured in nicotine-treated cultures; C, values measured in untreated cultures. The hatched portion of each bar represents immunoreactive Met-enkephalin contained in the cells and the unhatched portion the immunoreactive-Met-enkephalin released into the medium. The total height of each bar represents total (cell + medium) immunoreactive Met-enkephalin. Values are the mean $\pm$ s.e.m. of three determinations (wells). Lower s.e.m. is that associated with cell content and upper s.e.m. is that associated with total (cell + medium) immunoreactive Met-enkephalin.

Methods. Cells were prepared and maintained in culture at a density of 300,000 cells per 16-mm well as described previously⁴. On day 3 of culture, medium was removed and replaced with fresh medium (C) or medium containing 5 μ M nicotine (N). 24, 48 and 72 h later, parallel cultures were extracted for total nucleic acids or enkephalin peptides as described elsewhere⁵.

Table 1 Effect of hexamethonium blockade on nicotine-stimulated Met-enkephalin metabolism and increased cellular cyclic AMP in cultured chromaffin cells

1	2	3	4	5
Additions	mRNA ^{enk} % of control	Met-enkephalin (ng per well)	Met-enkephalin (% release)	Cyclic AMP (pmol per well)
None	100 $\pm$ 3.6	2.54 $\pm$ 0.211	1.55 $\pm$ 0.398	1.19 $\pm$ 0.025
Nicotine	214 $\pm$ 18.5*	3.40 $\pm$ 0.147*	22.0 $\pm$ 0.41*	1.73 $\pm$ 0.066*
Hexamethonium	128 $\pm$ 5.6	2.53 $\pm$ 0.190	1.63 $\pm$ 0.071	1.32 $\pm$ 0.178
Nicotine + hexamethonium	138 $\pm$ 5.1†	2.33 $\pm$ 0.287†	5.18 $\pm$ 0.398*†	1.43 $\pm$ 0.050*†

Parameters measured 24 h (column 2) or 48 h (column 3) after exposure to 10 μ M nicotine and/or 50 μ M hexamethonium (added to the cultures 15 s before addition of nicotine) or 1 h (columns 4, 5) after addition of 10 μ M nicotine with simultaneous addition of 10 μ M hexamethonium (when present). Cyclic AMP was measured with a commercially available kit (NEN) by direct radioimmunoassay. Values represent the mean $\pm$ s.e.m. of 3 (cols 2, 5), 4 (col. 4) or 6 (col. 5) determinations (wells).

* $P < 0.01$ compared with control (no additions); † $P < 0.05$ compared with nicotine alone, using Student's two-tailed *t*-test.

lished observations). Muscarine has no effect on enkephalin release or biosynthesis at concentrations up to 200 μ M (data not shown).

The induction of enkephalin mRNA by nicotine is preceded by an increase in intracellular cyclic AMP which is also blocked by hexamethonium (Table 1). We have previously reported that forskolin and cholera toxin, which increase intracellular cyclic AMP, also increase mRNA^{enk} in chromaffin cells^{5,6}. Elevation of intracellular cyclic AMP has also been demonstrated to result in increased transcription of the prolactin gene in a clonal line of pituitary tumour cells⁷. Interestingly, elevation of intracellular cyclic AMP by forskolin neither stimulates acute release of Met-enkephalin (or catecholamines) from chromaffin cells nor potentiates the releasing effect of a submaximal dose of nicotine (L.E.E., unpublished data), consistent with a previous report⁸ of a lack of effect of 8-bromo-cyclic AMP or cholera toxin on catecholamine release from cultured chromaffin cells. In addition, forskolin-induced enkephalin mRNA elevation is not

dependent on calcium influx whereas the increase in mRNA^{enk} elicited by 5 μ M nicotine is markedly so, being totally abolished in the presence of the calcium channel blocker D-600 (control mRNA^{enk}, $100 \pm 30.4\%$; 25 μ M forskolin, $237 \pm 15.6\%$; 25 μ M forskolin plus 10 μ M D-600, $290 \pm 25.3\%$; 5 μ M nicotine, $237 \pm 15.6\%$; 5 μ M nicotine plus 10 μ M D-600, $110 \pm 5.8\%$; data obtained from Northern hybridization and normalized as described in Fig. 1 legend). The calcium dependence of nicotinic induction of mRNA^{enk} may reflect a calcium requirement for activation of adenylate cyclase, as described for other systems in which secretagogues cause an elevation of intracellular cyclic AMP^{9,10}, or it may be necessary for the translocation of other messengers that ultimately affect enkephalin gene expression.

These data suggest a model for stimulus-secretion-synthesis coupling¹¹ in which secretagogue receptor activation causes both calcium-dependent secretion and a calcium-dependent increase in mRNA coding for the secreted peptide. The third messenger for stimulation of enkephalin biosynthesis may be cyclic AMP, acting via cyclic AMP-dependent protein kinase to enhance enkephalin gene transcription, as suggested for trans-synaptic induction of tyrosine hydroxylase in adrenal medulla^{8,12}.

It will be interesting to determine whether this mode of regulation also operates in other types of neural cells that store and secrete enkephalin as well as other neuropeptide hormones.

We thank Anna Iacangelo for the preparation of cDNA, Chang-Mei Hsu for carrying out the Met-enkephalin radioimmunoassays, Dr Steve Sabol for the gift of RB4 antiserum, Dr Peter Seeburg (Genentech) for the gift of bovine enkephalin plasmid, and especially Pat Thurston for the preparation of this manuscript and Michael Brownstein for his encouragement and advice.

Received 14 May; accepted 1 October 1984.

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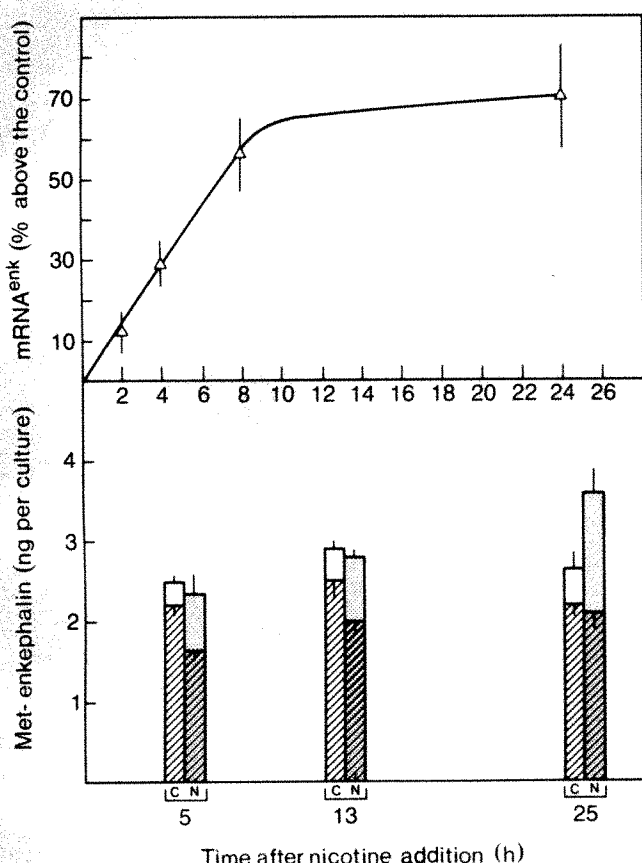


Fig. 2 *a*, Time course of mRNA^{enk} induction by nicotine; *b*, enkephalin immunoreactivity. The hatched and unhatched portions of the shaded and unshaded bars are as described in Fig. 1 legend.

Methods. Chromaffin cells were exposed to nicotine on day 3 of culture. At the times indicated, cultures were extracted for total nucleic acids or enkephalin peptides. *a*, Total nucleic acids were prepared as described previously^{5,6}, and the extract from 90,000 cells ($\sim 1 \mu$ g of RNA) in 100 μ l of denaturing buffer was blotted onto Gene-Screen Plus paper (NEN) soaked in 20 $\times$ SSC in a Schleicher and Schuell Slot Blot apparatus. Blots were washed with 1 M sodium phosphate pH 6.5, air-dried and hybridized with the bovine enkephalin cDNA probe (see Fig. 1 legend). Blots were autoradiographed and each sample was then cut from the paper and radioactivity counted by liquid scintillation spectrometry in 10 ml of Hydrofluor (NEN). Values are expressed as per cent increase over the untreated control signal at each time point, and represent the mean $\pm$ s.e.m. of three individual determinations (wells). *b*, Enkephalin immunoreactivity was measured in culture medium and cell extracts at 5, 13 and 25 h after exposure to nicotine. Values are the mean $\pm$ s.e.m. of six determinations (wells).

The yeast ubiquitin gene: head-to-tail repeats encoding a polyubiquitin precursor protein

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Ubiquitin¹, a 76-residue protein, occurs in cells either free or covalently joined to a variety of protein species^{2,3}, from chromosomal histones⁴⁻⁶ to cytoplasmic proteins. Conjugation of ubiquitin to proteolytic substrates is essential for the selective degradation of intracellular proteins in higher eukaryotes^{7,8}. We show here that a protein homologous to human ubiquitin exists in the yeast *Saccharomyces cerevisiae*, and that yeast extracts conjugate human ubiquitin to a variety of endogenous proteins in an ATP-dependent reaction. We have isolated the *S. cerevisiae* ubiquitin gene and found it to contain six consecutive ubiquitin-coding repeats in a head-to-tail arrangement. This apparently unique gene organization suggests that yeast ubiquitin is generated by processing of a precursor protein in which several exact repeats of the ubiquitin

amino acid sequence are joined directly via Gly-Met peptide bonds between the last and first residues of mature ubiquitin, respectively. Ubiquitin-coding yeast DNA repeats are restricted to a single genomic locus; although the sequenced repeats differ in up to 27 of 228 bases per repeat, they encode identical amino acid sequences. As this predicted amino acid sequence differs in only 3 of 76 residues from that of ubiquitin in higher eukaryotes, ubiquitin is apparently the most conserved of known proteins.

We identified recently the first mutant in the ubiquitin pathway⁹, and have used this mouse-derived cell cycle mutant, ts85, to directly demonstrate the ubiquitin dependence of selective protein degradation in higher eukaryotes⁷. A deeper analysis of the ubiquitin pathways by methods of molecular genetics should be possible if yeast possesses a functional ubiquitin system. Figure 1A shows that yeast contain a protein homologous to ubiquitin. This protein is specifically immunoprecipitated with an antibody to human ubiquitin, and is indistinguishable from ubiquitin when analysed electrophoretically on 18% polyacrylamide-SDS gels (Fig. 1A). Moreover, crude *S. cerevisiae* extracts catalyse the conjugation of added human ubiquitin to a variety of endogenous proteins in an ATP-dependent reaction (Fig. 1B, lanes d-h), as do mammalian cell-free systems (Fig. 1B, lane c) and intact cells^{7,9-12}.

The recently developed λ gt11 expression vector^{13,14} is suited for cloning genes whose products can be visualized by filter immunoassay. A yeast genomic DNA library carried in λ gt11 was screened using affinity-purified antibody against human ubiquitin, and a specifically immunoreactive phage, λ UB, was plaque-purified to homogeneity (see Fig. 2A legend for details). The nucleotide sequences at both ends of the λ UB insert were determined using the method of Maxam and Gilbert¹⁵. However, we found stop codons in all three reading frames and no potential ubiquitin-coding sequences. This suggests that vector-insert fusion proteins were not responsible for recognition by the anti-ubiquitin antibody.

To identify the immunoreactive coding sequences of λ UB, its 2.4 kb insert was sheared by sonication and subcloned into the pUC13 plasmid expression vector¹⁶ (Fig. 2A). An immunoreactive subclone designated pUB1 was purified, and the nucleotide sequence of its 240 base pair (bp) insert was determined. As shown in Fig. 2A, this DNA fragment does indeed encode an amino acid sequence identical to the C-terminus of human ubiquitin, from residue 44 to the terminal residue 76. Surprisingly, however, the predicted amino acid sequence continues without stop codons far past the presumptive ubiquitin C-terminus; these downstream sequences correspond in turn to the N-terminus of human ubiquitin (Fig. 2A).

The striking organization of the pUB1 insert (Fig. 2A) suggests that yeast ubiquitin is generated by processing of a precursor protein containing two or more precisely joined repeats of the canonical amino acid sequence of ubiquitin. Using the pUB1 insert as a probe, ubiquitin-coding sequences of the λ UB insert were mapped by Southern hybridization¹⁷ (data not shown), and found to span approximately 1.4 kb internal to the 2.4 kb λ UB insert, consistent with the possibility that λ UB contained a string of six 228-bp ubiquitin-coding repeats. The λ UB insert was subcloned into pBR322 (to yield the plasmid pUB2; see legend to Fig. 3), end-labelled as shown in Fig. 3A, and partially digested with either *Msp*I or *Mbo*II. This produced an electrophoretic 'ladder' of end-labelled products, as predicted by the model of multiple, precisely joined repeats of the 228-bp ubiquitin-coding elements (Fig. 3B and data not shown; see also Fig. 2A, B). Based on such restriction site periodicities, the ubiquitin gene appears to contain six tandem ubiquitin-coding repeats. An independent estimate of six repeats can be made on the basis of Southern hybridization analysis of either *S. cerevisiae* genomic DNA or the 2.4 kb pUB2 insert fully digested with *Dde*I and probed with pUB1 insert (see Fig. 3C). Figure 3C also shows that no major rearrangements of ubiquitin-coding sequences occurred during cloning of the pUB2 insert from yeast genomic DNA. A quantitative hybridization analysis, using DNA from λ UB as a titration standard, indicates that

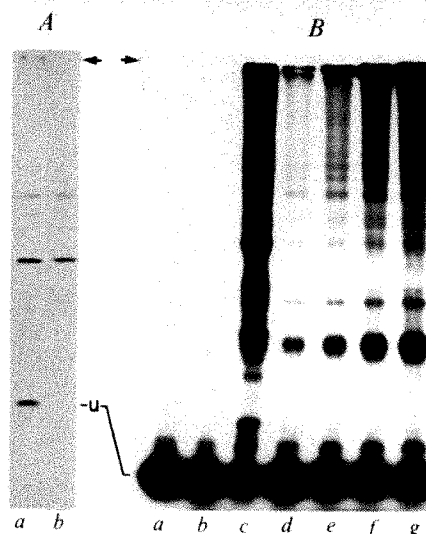


Fig. 1 Presence of a ubiquitin homologue in *S. cerevisiae*, and ATP-dependent conjugation of human ubiquitin to endogenous proteins in *S. cerevisiae* extracts. **A**, yeast cultures (strain S288C) in exponential growth were labelled with ³H-lysine in glucose-supplemented SD medium for 30 min at 30 °C. Cells were pelleted by centrifugation, resuspended in 1 mM dithiothreitol (DTT), 10 mM Tris-HCl (pH 7.5), lysed using glass beads, and centrifuged at 100,000g for 1 h. The supernatant was heated at 80 °C for 10 min and clarified by centrifugation. Equal aliquots of the supernatant were immunoprecipitated (using fixed *S. aureus*³⁰) with either rabbit antiserum against human ubiquitin or an equal amount of preimmune rabbit serum (lanes a and b, respectively). Immunoprecipitates were analysed by electrophoresis in an 18% discontinuous polyacrylamide-SDS gel and fluorography⁹. Identical results were obtained in the absence of heat pretreatment, i.e., no additional protein species were specifically immunoprecipitated by the anti-ubiquitin antibody. u denotes the ubiquitin band. **B**, Strain S288C cells in exponential growth were lysed and centrifuged as above, and the supernatant used as a source of ubiquitin-conjugating activity. 120 µg of extract were assayed at 37 °C in a final volume of 30 µl by adding 3 µl (5 × 10⁵ c.p.m.) of human ubiquitin (purified and labelled with ¹²⁵I as described previously⁹) to the assay buffer (10 mM creatine phosphate, 0.5 mM ATP, 100 µg ml⁻¹ creatine phosphokinase, 5 mM MgCl₂, 1 mM DTT, 50 mM Tris-HCl, pH 7.5). Assays were terminated by addition of SDS⁹. Samples were analysed by electrophoresis on discontinuous 12.5% polyacrylamide-SDS gels. Lane a, from a mock assay (extract omitted); lane b, a complete yeast assay but in the absence of ATP (and presence of 0.6 units of yeast hexokinase and 20 mM 2-deoxyglucose); lane c, an analogous 6 min assay with ¹²⁵I-ubiquitin and 120 µg of rabbit reticulocyte extract prepared as described previously¹⁰. Lanes d-g, complete yeast assays carried out for 3, 6, 15 and 30 min, respectively. Arrows indicate the electrophoretic origins.

nucleotide sequence homology to the pUB1 insert is restricted in *S. cerevisiae* to a single genomic locus (Fig. 3D).

While the data of Fig. 3, based on restriction fragment analyses, adequately demonstrate the existence of multiple repeats of yeast ubiquitin-coding elements, the precise juxtaposition of these ubiquitin repeats (Fig. 2A) was shown to be general by more extensive nucleotide sequence determination (see Fig. 2B). Since the ubiquitin-coding repeat is of a size (228 bp) appropriate for sequence determination from a single site, the ubiquitin-coding region could be sequenced by placing each repeat next to a universal sequencing primer site, such as that of the mp family of M13-derived cloning vectors¹⁸. The desired M13 subclones correspond to the restriction fragments visualized autoradiographically in Fig. 3B. In a preparative counterpart of the same experiment, DNA was eluted from gel slices corresponding to the autoradiographic bands in Fig. 3B, and the fragments of interest were cloned selectively into the M13mp9 vector¹⁸ (see Fig. 2B legend).

Sequencing of the nested set of ubiquitin-coding repeats, subcloned into M13 as described above, substantiated in detail

Fig. 2 Nucleotide sequences of the yeast ubiquitin gene. Arrowheads indicate sites of proteolytic cleavage required to generate mature ubiquitin from a polyubiquitin precursor; predicted amino acids are thus numbered from 1 to 76 beginning with predicted mature amino termini (see Fig. 4 and text). **A**, Complete nucleotide sequence of pUB1 insert. **B**, nucleotide sequences spanning the 3' end of the yeast ubiquitin gene.

Methods: **A**, The plasmid pUB1 contains a 240 bp insert subcloned from the λ UB phage. λ UB was isolated as described in the text from a yeast genomic DNA library carried in the λ gt11 vector¹³ (for preparation of the library see ref. 14). The polyclonal antibodies against human ubiquitin were affinity-purified on ubiquitin-Sepharose before immunoscreening at a dilution optimized in preliminary dot immunoassays. These antibodies were negative with *E. coli* extracts. Screening of the library (carried out via plaque immunoassay, at 5×10^5 plaques per 15-cm plate, with the host *E. coli* Y1090, as described previously¹⁴) yielded fifty apparently positive phage stocks. Two of four such stocks, tested further, produced immunological signals specifically inhibitable by added ubiquitin (not shown). One such phage, λ UB, was plaque-purified until each visible plaque (of about 250 tested) was immunoreactive. The immunoreactive protein specific to λ UB lysates is ~50 kilodaltons in size, and its expression is IPTG-independent (data not shown). DNA from the cloned λ UB phage was digested with *Eco*RI to yield the 2.4 kb λ UB insert, which was then purified by gel electrophoresis and sheared by sonication to an average size of ~400 bp. The resulting DNA fragments were subcloned into the pUC13 plasmid expression vector¹⁶ using synthetic linkers. The pUC13 library was immunoscreened with the anti-ubiquitin antibody, using previously described methods³¹; one of the positive clones, designated pUB1, was purified and its insert was sequenced on both strands, using the method of Maxam and Gilbert¹⁵. **B**, The insert of λ UB was excised with *Eco*RI and subcloned into the *Eco*RI site of pBR322, to yield the pUB2 plasmid. pUB2 was cut with *Hind*III (see Fig. 3A), and a small portion of the resulting DNA sample was ³²P end-labelled with the Klenow DNA polymerase I fragment³², to serve as a tracer, then added back to the same DNA sample. The resulting mixture of labelled and unlabelled DNA was then cut with *Pst*I (see Fig. 3A). The insert-containing fragment was purified by agarose gel electrophoresis, partially digested with *Msp*I, treated with bacterial alkaline phosphatase, and analysed on a 3% polyacrylamide gel (see Fig. 3B). The wet gel was subjected to autoradiography, and DNA was eluted from gel slices, each corresponding to an autoradiographic band. The resulting DNA samples were ligated in parallel to the *Hind*III site of replicated form DNA of M13mp9 phage¹⁸. The vector DNA had been previously digested with both *Hind*III and *Pst*I, so that only one *Hind*III site was available for ligation. Following heat-inactivation of DNA ligase, the samples were digested with *Acc*I to provide a single-stranded end on the vector which is complementary to the *Msp*I-generated single-stranded end of the inserted fragment. Ligation products of the desired size were then purified on an agarose gel, recircularized via *Acc*I-*Msp*I ligation, and transformed into *E. coli* JM101. Phages from plaques which appeared white on plates containing Xgal and IPTG were amplified, and replicative form DNA was prepared and digested with various restriction enzymes to verify its expected identity. Only the expected M13 subclones were obtained using the two-step ligation scheme described above, that is, all subclones had inserts of the same size as their 'parental' restriction fragments, and the subcloned inserts were specifically oriented, with ubiquitin-coding sequences proximal to the universal sequencing primer site. This site was used for sequencing roughly 280 nucleotides into each of the overlapping classes of the M13 subclones; the sequence in Fig. 2B was compiled from four such sets of overlapping sequence data. Phage DNA was sequenced by the chain termination method as previously described³³, using [³⁵S- α]dATP (ref. 34). The 'non-ubiquitin' Asn residue encoded at the end of ubiquitin gene is boxed. Differences between the nucleotide sequences of the two ubiquitin-coding repeats nearest to the end of the coding sequence are indicated in the last repeat by shadowing. Note that all of the sequenced repeats encode identical amino acid sequences. A nucleotide sequence which resembles the consensus sequence for transcription termination in yeast¹⁹ is indicated by dots.

the concept of a polyubiquitin precursor protein (Fig. 2B). The sequenced portion of the ubiquitin gene domain includes three and a half ubiquitin-coding repeats and three head-to-tail repeat junctions, each of which encodes Gly-Met, a putative site of processing, as inferred originally from the sequence of the pUB1 insert (Fig. 2A, B). A nucleotide sequence resembling the consensus sequence for transcription termination in yeast¹⁹ is found 160 bp downstream from the stop codon of the ubiquitin gene (Fig. 2B).

Remarkably, only the last of the sequenced ubiquitin-coding repeats encodes 77 rather than 76 residues, characteristic of mature ubiquitin (Fig. 2B). The extra residue, Asn, is at the end of a putative polyubiquitin precursor protein where it immediately follows the C-terminal Gly residue of mature ubiquitin (Fig. 2B). By blocking the carboxy-terminus of the preceding Gly residue, the 'non-ubiquitin' C-terminal Asn could serve to prevent participation of unprocessed polyubiquitin in reactions of ubiquitin-protein conjugation. The properties of recently characterized ubiquitin-specific hydrolases from mammalian

A

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44      Msp I      Mbo II      63
... ATC TTT GGC GGT AAG CAG CTA GAA GAC GGT AGA ACC TTG TCT GAC TAC AAC ATC CAA AAG
   ile phe ala gly lys gln leu glu asp gly arg thr leu ser asp tyr asn ile gln lys

64      76 1      7
CAA TCT ACT CTT CAC TGC GTG TTG AGA TTG AGA GGT GGT ATG CAA ATT TTT GTC AAC ACA
glu ser thr leu his leu val leu arg leu arg gly gly met gln ile phe val lys thr

8      27
CTG ACA GGT AAG ACT ATA ACC CTA GAG GTT GAA TCT TCT GAC ACT ATT GAC AAC GTT AAG
leu thr gly lys thr ile thr leu glu val glu ser ser asp thr ile asp asn val lys

28      45      Msp I
TGC AAA ATT CAA GAC AAG GAA GGT ATT CCT CCA GAT CAA CAA AGA TTG ATC TTT GGC GGT ...
ser lys ile gln asp lys glu gly ile pro pro asp gln gln arg leu ile phe ala gly

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B

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39      Msp I      Mbo II      59
... GAT CAA CAG AGA TTG ATT TTT GGC GGT AAG CAA CTA GAA GAT GGT AGA ACC TTG TCT GAC TAC
asp gln gln arg leu ile phe ala gly lys gln leu glu asp gly arg thr leu ser asp tyr

60      76 1      4
AAC ATC CAA AAG GAA TCT ACT CTT CAC TTG GTG TTG AGA CTC AGA GGT GGT AGA CAA ATT TTC
asn ile gln lys glu ser thr leu his leu val leu arg leu arg gly gly met gln ile phe

5      25
GTC AAA ACT CTA ACA GCG AAG ACT ATA ACC CTA GAG GTT GAA TCT TCC GAC ACT ATT GAC AAC
val lys thr leu thr gly lys thr ile thr leu glu val glu ser ser asp thr ile asp asn

26      Msp I      46
GTC AAA AGT AAA ATT CAA GAT AAA GAA GGT ATC CCT CCG GAT CAA CAG AGA TTG ATT TTT GCT
val lys ser lys ile gln asp lys glu gly ile pro pro asp gln gln arg leu ile phe ala

47      Mbo II      67
GGT AAG CAA CTA GAA GAT GGT AGA ACC TTG TCT GAC TAC AAC ATC CAA AAG GAA TCT ACT CTT
gly lys gln leu glu asp gly arg thr leu ser asp tyr asn ile gln lys glu ser thr leu

68      Dde I      76 1      12
CAC TTG GTG TTG AGA CTC AGA GGT GGT ATG CAA ATT TTC GTC AAA ACT CTA ACA GGC AAG ACT
his leu val leu arg leu arg gly gly met gln ile phe val lys thr leu thr gly lys thr

13      33
ATA ACC CTA GAG GTT GAA TCT TCC GAC ACT ATT GAC AAC GTC AAG GAA AAA ATT CAA GAC AAG
ile thr leu glu val glu ser ser asp thr ile asp asn val lys ser lys ile gln asp lys

34      Msp I      Mbo II      54
GAA GGT ATT CCT CCA GAC CAA CAA AGA TTG ATC TTT GGC GGT AAG CAA CTA GAA GAT GGT AGA
glu gly ile pro pro asp gln gln arg leu ile phe ala gly lys gln leu glu asp gly arg

55      75
ACG CTG TCG CAC TAC AAT ATT CAA AAG GAG TCG ACT CTT CAC CTC GTC TTG ACG CTC AGG GGT
thr leu ser asp tyr asn ile gln lys glu ser thr leu his leu val leu arg leu arg gly

76 1
GGT AAC TGA TCACTCTCGCAATATTTTCATTATGTCAATATATATATTTACTCTCTCTTTTGGTTTTTTTTTTT
gly asn stop

TTTGATAAATACCTCCATAGAACACTAAATAAATTTGTTCACTGTGTTATTTGCTTTATTCATGTTGGTTTTCAGAGCTTGG
TTTTGAATCGCTTATATATATACCTTCACTATTTTCGGCAACCCGGGAATACCTAGCTATTTTGATAGAAAGGATTTT
ATTAGGGAATAAACCACAATTAAGGTGCTCATCTGTTTCACTCCAGTAACGACATACCGCCCAATTCAGCTCAA
CCTTTTAACTCTATTATTAATTGATACGGATAGAATATTCTGACTACCAAAAGGGAAGGCAAAAAAGG...

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cells²⁰⁻²² are compatible with their involvement in processing of polyubiquitin via cleavages at Gly-Met and Gly-Asn junctions. Complete sequencing of the ubiquitin gene domain (now under way) should show whether the putative polyubiquitin precursor contains any non-ubiquitin amino acid sequences other than the C-terminal Asn residue (Fig. 2B). Preliminary sequencing information (not shown) strongly suggests that the N-terminus of the putative polyubiquitin is identical to that of mature ubiquitin, and confirms the above estimate of sixfold repetition of ubiquitin-coding elements.

The polypeptide organization of the yeast ubiquitin gene is novel because the polypeptides previously described are all either secretory^{23,24} or viral²⁵ gene products, and contain 'spacer' sequences discarded in processing. Furthermore, with the exception of protein precursors to yeast mating factors, which in the case of α -factor^{26,27} contain four identical repeats of the mature α -factor sequence separated by six to eight residue long spacers, no other known polypeptides are internally repetitive to a comparable extent.

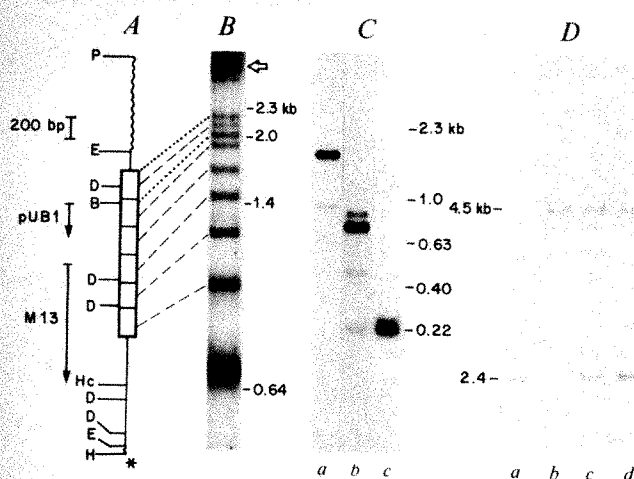


Fig. 3 Structural analysis of yeast ubiquitin-coding sequences. **A**, diagrammatic representation of an insert-containing restriction fragment from the plasmid pUB2 (see text and legend to Fig. 2B) cut with *Pst*I (top) and *Hind*III (bottom). The boxed segment indicates the ubiquitin-coding domain; junctions between ubiquitin-coding repeats are shown as five horizontal lines within the box. These lines correspond to the arrowheads in Fig. 2. The sequenced portions of the insert (see Fig. 2) are indicated at the left by arrows which also denote the predicted direction of ubiquitin gene transcription. Restriction endonuclease sites: P, *Pst*I; E, *Eco*RI; D, *Dde*I; B, *Bgl*II; Hc, *Hinc*II; H, *Hind*III. The asterisk at the bottom right marks the end-labelling site used in panel B (see also Fig. 2B). Wavy lines indicate vector DNA. **B**, periodic distribution of *Msp*I restriction sites in the cloned yeast ubiquitin gene. **C**, structural analysis of ubiquitin-coding sequences in yeast genomic DNA.

Methods: **B**, The plasmid pUB2 (see text and legend to Fig. 2B) was cut with *Hind*III, ³²P-end-labelled with Klenow DNA polymerase I fragment²², then cut with *Pst*I. The insert-containing, end-labelled fragment was purified by agarose gel electrophoresis, partially digested with *Msp*I and analysed by electrophoresis on a 2% agarose gel followed by autoradiography (panel B). Open arrow (top), position of the end-labelled fragment uncut by *Msp*I. This uncut fragment is diagrammed in panel A. The dashed lines between panels A and B point to positions of *Msp*I cleavage sites on the physical map of panel A, and connect these sites to the corresponding radiolabelled fragments seen in panel B. Dotted lines are used to indicate a pair of *Msp*I sites present in addition to the major set of repetitive *Msp*I sites. **C**, Genomic DNA from strain S288C was digested with restriction enzymes, electrophoresed on a 2.0% agarose gel, transferred to Gene Screen (NEN) and hybridized³⁶ using gel-purified insert from pUB1 as a probe. Restriction enzymes: lane a, *Bgl*II and *Hinc*II; lane b, *Dde*I; lane c, *Msp*I. An estimate of six ubiquitin-coding repeats is confirmed by the *Dde*I digest (lane b): fragments at 0.8 and 0.23 kb each contain exactly one repeat (see Figs 2B, 3A), and the prominent fragment at 710±20 bp spans just over 3 repeats, by estimate either of size or relative hybridization intensity, leaving, again by either of these criteria, ~2/3 of a repeat associated with the 0.45 kb fragment (see also Fig. 3A). **D**, ubiquitin-coding yeast DNA sequences are present at a single genomic locus. DNAs purified from the *S. cerevisiae* strain A364A (the same results were obtained with strain S288C) and from the λUB phage (see Fig. 2) were digested with *Eco*RI, mixed in varying proportions and electrophoresed on a 0.8% agarose gel, followed by Southern hybridization with the purified ³²P-labelled insert of pUB1, which contains exclusively ubiquitin-coding nucleotide sequences (see Fig. 2A). Only the hybridization pattern is shown. Lane a, 8.5 ng (one molar equivalent) *Eco*RI-cut λUB DNA; lane b, 2.5 μg (one molar equivalent) *Eco*RI-cut yeast DNA; lane c, 2.5 μg yeast DNA plus 8.5 ng λUB DNA (1:1 molar ratio); lane d, 2.5 μg yeast DNA plus 17 ng λUB DNA (1:2 molar ratio). Molecular weight markers (not shown) were used to deduce the lengths of ubiquitin gene-specific DNA fragments.

human:	1	met-gln-ile-phe-val-lys-thr-leu-thr-gly-lys-thr-ile-thr-leu-glu-val-glu-pro	19
yeast:	...	...	ser
human:	20	ser-asp-thr-ile-glu-asn-val-lys-ala-lys-ile-gln-asp-lys-gly-ile-pro-pro	38
yeast:	...	asp	ser
human:	39	asp-gln-gln-arg-leu-ile-phe-ala-gly-lys-gln-leu-glu-asp-gly-arg-thr-leu-ser	57
yeast:	...	...	...
human:	58	asp-tyr-asn-ile-gln-lys-glu-ser-thr-leu-his-leu-val-leu-arg-leu-arg-gly-gly	76
yeast:	...	...	...

Fig. 4 Comparison of the deduced amino acid sequence of yeast ubiquitin with that of ubiquitin in higher eukaryotes. The sequence given for human ubiquitin is identical to that of bovine, trout and insect ubiquitin^{1,3,35}. The amino acids of human ubiquitin are numbered from 1 to 76, beginning with its N-terminus, as defined by amino acid sequence data¹.

Another interesting feature of the ubiquitin-coding repeats is that although the sequenced repeats differ in up to 27 out of 228 bases per repeat (Fig. 2B), they encode identical amino acid sequences (Figs 2 and 4). The predicted amino acid sequence of yeast ubiquitin differs in only 3 of 76 residues from that of ubiquitin in higher eukaryotes (Fig. 4). This striking evolutionary stability exceeds that of histones^{28,29} and other known proteins.

Finally, recent experiments indicate that in mouse ts85 cells, which have a thermolabile ubiquitin activating enzyme⁹, a putative polyubiquitin precursor protein is strongly induced at the nonpermissive temperature (D.F. and A.V., unpublished data). Thus, the apparently unique organization of the yeast ubiquitin gene discovered in the present work may be characteristic also of ubiquitin genes in higher eukaryotes. Although the functional significance of the polyubiquitin gene organization is not yet understood, a number of competing hypotheses may now be addressed directly using the methods of yeast genetics.

We thank Richard Young and Ronald Davis for the Agt11 library, Aaron Ciechanover for the anti-ubiquitin antibody, Jacqueline Whang for her assistance in some experiments, and Igor Roninson, Mark Solomon, François Strauss, Paul Swerdlow and especially Joan Park and Lulu Pickering for technical advice and comments on the manuscript. We also thank Barbara Doran for secretarial assistance. This work was supported by grants to A.V. from the National Institute of General Medical Sciences (GM31530 and GM26086).

Received 12 September; accepted 15 October 1984.

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Biotechnology bookshelf

Anita Crafts-Lighty's choice of the best biotechnology reference books published in 1984.

AFTER a flurry of activity over the past two to three years, the rate of appearance of new books dealing with biotechnology seems to have slowed down a little in 1984. This is particularly evident in the reference department. Nevertheless there have still been a few landmark publications which all biotechnologists should know about and few libraries should be without. Those described below are a personal (and far from comprehensive) selection of the best biotechnology reference books published during this year.

Best basics

PUBLIC awareness of biotechnology is growing steadily and at last there is a readable, accurate, up-to-date and balanced introductory account which can be recommended whole-heartedly to one's non-scientist colleagues. It is cheating a little to include Steve Prentis's *Biotechnology — A New Industrial Revolution* (Orbis, London/ George Braziller, New York; £9.99, \$18.50) under the heading of a reference work, but this is an excellent book to turn to for clear, simple explanations of the complex scientific achievements of molecular biology. However Prentis also goes well beyond genetic engineering to look at many other aspects of biotechnology and their impact on society. The reader cannot help being caught up in the author's manifest enthusiasm for his subject matter, but at the same time one can note with relief an absence of hyperbole and very few errors of fact.

Bergey's bugs

FEW microbiologists will not know of *Bergey's Manual of Determinative Bacteriology*, but anyone who has bought a copy in the past few years may have been surprised to notice that the current (eighth) edition was actually published in 1974. A *Shorter Bergey's Manual of Determinative Bacteriology* did come out in 1977 followed, in 1981, by a supplement to the 1966 taxonomists' handbook *Index Bergeyana*, but these were only stop-gaps and a new edition of the basic work has been badly needed for some time. To rectify the situation, Vol.1 of a massive new work, *Bergey's Manual of Systematic Bacteriology*, was published earlier this year (Williams and Wilkins; \$80, £77). John G. Holt is editor-in-chief, but the first volume contains contributions from more than 125 scientists. In over 950 pages, 11 groups of bacteria are covered (spirochaetes, aerobic motile gram-negatives, motile curved gram-negatives, gram-negative aerobic rods, anaerobic rods,

sulphate/sulphur-reducing bacteria, anaerobic gram-negative cocci, rickettsia and chlamydia, mycoplasmas and endosymbionts). Presumably gram-positives, spore-formers and others will be dealt with in Vol.2, which is scheduled to appear late next year. The first volume also has sections on taxonomy and nomenclature, a list of culture collections, an extensive (105-page) bibliography and an index. Its scope is far wider than the determinative manual although much similar material is included. This mammoth tome is not exactly light reading, but the book is unquestionably the most useful and detailed compendium of microbiological information available and a must for every library serving microbiologists.

Boring but better sequences

Nucleotide Sequences 1984 (IRL Press; £50, \$75) must rate as the most boring biotechnology reference book of 1984 to read through; but that is not what it is designed for. This paperback work of 1,221 pages, divided between two volumes, lists over 4,000 reported nucleic-acid sequences containing nearly three million bases. It is the first printed compilation of sequences in the European Molecular Biology Laboratory's Nucleotide Sequence Data Bank (Release 3.0) and the Gen Bank Genetic Sequence Data Bank (Release 17.1). Since the databases are updated periodically, the computer systems will often contain more information than the books, and, of course, these databases can be searched in more sophisticated ways than a printed compilation. But for those without computer access or for the occasional "Has this gene been sequenced?" enquiry, the printed compendium will be of great value. The entire text is reproduced from a computer printout, which leads to admirably concise entries, though there is still room for better presentation in a number of minor ways. For example authors' names should surely be capitalized. Sequences are grouped by source (mammalian, other vertebrates, invertebrates, plants, organelles, bacteria, structural RNA, viruses, bacteriophages, synthetic and so on) and there are author and keyword phrase indexes, plus indexes relating the "entry names" used in the databases to the accession numbers used in the book.

Biotech business

ONE of the most widely quoted biotechnology documents of 1984 is the United States Office of Technology Assessments' report *Commercial Biotechnology — An International Analysis* (US Government

Printing Office \$20). For those concerned with science policy and history, this is an invaluable reference tool, although it contains far fewer handy market figures than the OTA's 1981 report, *The Impacts of Applied Genetics*. Obviously, the publication is aimed primarily at an American audience, and it is therefore somewhat biased in its outlook, but it still represents an excellent in-depth summary of the position of commercial biotechnology worldwide at the beginning of 1984.

British biotech directory

IN the interests of strengthening British biotechnology, the Biotechnology Unit of the Laboratory of the Government Chemist (part of the United Kingdom's Department of Trade and Industry) last year commissioned a directory covering British biotechnology companies and academic institutes. The result is *The 1984 Directory of British Biotechnology* (Cartermill Publishing, PO Box 33, St Andrews, Fife, UK; £48.50). Venture capital firms, contract research organizations, equipment suppliers and engineering contractors are all included, but the coverage of university laboratories and research institutes is somewhat more limited. The entries are accurate and quite up to date, and the book is nicely presented and easy to read. A perfectionist might ask for longer profiles, and a clearer distinction between product producers and contract research organizations, but these are minor failings in what is one of the best reference books of its type.

And the future?

THE ratio of commercially-orientated to scientifically-orientated books in this selection is probably indicative of the needs of biotechnology tomorrow. Looking towards 1985 and beyond, I would like to see more attempts to describe and catalogue the numerous cloned genes, expression vectors and monoclonal antibodies around, and more opinions of the strength of the patents being carefully filed by so many researchers and companies. Some comprehensible books on regulatory affairs, and more down-to-earth advice on the effective management of innovations and on academic liaison techniques, would be welcome too. Downstream processing, scale up and plant genetic engineering are also important areas of biotechnology where scientific advances are speeding up but the reference shelves remain too bare.

Many claim that biotechnology will be the most important industry of the twenty-first century and certainly research progress continues to astound — it should hold an equally bright future for support activities such as publishing, and that will be to the benefit of all concerned. □

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Who does what, and where?

John Galloway

Who's Who in Science in Europe: A Biographical Guide in Science, Technology, Agriculture, and Medicine, 4th Edn.

Longman/Gale Research: 1984. Three volumes, pp.2,556. £295, \$500.

The Medical Research Directory.

Edited by Jean Elizabeth Levy.

Wiley: 1983. Pp.730. £85, \$170.

Medical Research Centres: A World Directory of Organizations and Programmes, 6th Edn.

Consultant editors Leslie T. Morton and Jean F. Hall.

Longman/Gale Research: 1984. Two volumes, pp.1,087. £175, \$320.

World Guide to Scientific Associations and Learned Societies, 4th Edn.

Edited by Barbara Verrel and Helmut Opitz.

K.G. Saur Verlag, Munich and New York (distributed in Britain by Library Association Publishing, 7 Ridgmount Street, London): 1984. Pp.947. DM268, \$112, £80.

SCIENCE, we all appreciate, is *public* knowledge and its most apparent property, other than being quite inscrutable to members of the public and largely disassociated from them, is how much of it there actually is. UNESCO and the International Council of Scientific Unions recognized the problem when in 1971 they published a feasibility study on a World Science Information System. Twenty-two recommendations were made but they did not include registers of scientists or even laboratories. At least not explicitly. But a number of publishers have thought it worth a try.

Where does one start in trying to assess a small sample of such registers? First of all, is it possible to get at the raw numbers of scientific and technological manpower? Well, yes, up to a point. UNESCO's office of statistics produces world-wide figures on these, though the last tables I could get were published in 1980. The total world scientific and engineering manpower comes out at about 33 million, but the sorts of figures here do not simply refer to active research scientists publishing their findings in the open literature. Ominously, Britain doesn't feature in the analysis (I didn't realize things were so bad; perhaps no one could be bothered to fill in the form when it came round).

Suppose you are interested in more than just numbers — you want to know what the quality is like. This is even trickier. You can go for members of the various national academies of science, for example, where membership is by election on grounds of merit — not foolproof of course. After that you are firmly in *Who's Who* land and for the purpose of this review *Who's Who in Science in Europe*. This used to be published by Francis Hodgson but the latest edition comes from Longman. There are 25,000 scientists named (compare this with the UNESCO figure of 4,800,000, excluding Britain), with biographical details on about half of them. The rather similar *American Men and Women of Science* lists 130,000, about

one in twenty of their UNESCO total.

Well, who *is* who in European science? Quite clearly it is the heads of laboratories and of the larger university departments. The compilation started with Longman's own two-volume *European Research Centres* which seems to list about 18,000 government laboratories, departments spending more than £50,000 a year, and some public and private laboratories. This was supplemented by an exercise based upon details of most of the authors of scientific books in the past five years, and also by sending out 50,000 enquiry forms to other senior scientists of whom about a quarter actually replied. Those who did not respond, but for whom some information was available, end up as brief entries. So the two problems of compiling this sort of reference list are revealed — first how to choose who to seek information from, and then to get a decent rate of replies. The consequence is that the quality of the information is rather variable. In the field of neuropharmacology it is in fact misleading — neither Neher or Sakmann (the inventors of patch clamping) figure, Rang's move from the chair of Pharmacology at University College London to be Director of the new Sandoz Institute is not recorded, and Leslie Iversen appears twice, first as L.L. and Director of the MRC's Neuropharmacology Unit in Cambridge and then as Lars Leslie and Director of the new Merck, Sharp and Dohme Neurosciences Institute — someone should have picked that up, even given the long gestation period of such books.

The next question is who consults something like this. Cynically I thought it was the scientists whose names are included. Longman cheerfully admit a cheap offer to those who appear was part of the pitch, but in the end they expect to sell about 1,500 copies to libraries, mostly in Europe but a fair proportion through Gale in the United States. Librarians I spoke to — admittedly not a large sample — would very much like to have it simply as a guide to the more successful and still

relatively active scientists. And for Europe there really isn't anything else. Still, it would be nice to have a *Good Scientists Guide* as well as one for top scientists, that is to say scientists who have achieved administrative positions. This one is really just a guide to personalities. Lack of anything but a rudimentary index prevents it from being anything more.

If you simply want to associate scientists and subjects, the best way into the labyrinth is through the *Science Citation Index* and the *Permuterm Subject Index* which are available as computerized databases. The only biographical database is *American Men and Women of Science*, available through DIALOG; there is nothing for Europe.

So much for who, what about where? If you do want a more detailed picture of science and the scientists doing it then the price that has to be paid is a narrowing of scope — of subject or geography, or both. For the Commonwealth there is the *Commonwealth Universities Yearbook*, in four volumes, a bald listing of the staff members in universities from Australia to Zimbabwe. In Britain the British Library publishes annually the three-volume *Research in British Universities, Polytechnics and Colleges* (RBUPC). The 1984 edition is just out and seems pretty comprehensive with nearly 50,000 scientists mentioned and a detailed breakdown of research by department, broad subject heading and project. (In France this sort of information is available on the Labinfo and CNRS databases.) A further narrowing by subject is apparent in Wiley's *Medical Research Directory* which uses a breakdown very similar to the RBUPC. It features the research of 650 different institutes, using 45 broad subject headings, subdivided first by departments and then by projects. The index contains 17,000 names and 22,000 terms.

In principle you might penetrate British medical research with this volume and identify scientists with particular research interests. On the other hand the breakdown by subject is whimsical. Neuroscience gets only four pages. It includes, correctly, three research divisions at the National Institute for Medical Research but gets only four out of 21 of the MRC neurosciences units. Leslie Iversen features again as Director of the Neurochemical Pharmacology Unit in Cambridge — he resigned some eighteen months ago; and half of the Unit's senior scientists mentioned are no longer there. Dr J.B. Brierley, a neuropathologist stated as being at the MRC Laboratories at Carshalton, has been retired for at least three years. The main flaw, obviously, is that much of what is listed is undeniably out of date. To be quite fair the publishers say they have deliberately included recent as well as current research, but that does not really draw the teeth of the criticism. And given the magnitude of this problem, it is almost

surprising that publishers try to compile such directories at all. What they should do of course — and what in fact Wiley do now do — is to provide the information through on-line databases. These are much easier to keep up to date and can be searched far more effectively than their printed counter parts.

The price paid for broadening the geography, while keeping the general scope, is a loss of detail. Longman's *Medical Research Centres* covers 11,000 laboratories and organizations including charities, commercial laboratories and individual university departments worldwide, arranged alphabetically and by country. A broad idea of the interests of each one can be obtained, quite often together with the yearly budget. Commercial laboratories seem a bit coy about theirs. Again in those areas where I had detailed, up-to-date information, I found these volumes tended to be out-of-date — and sometimes things were not spelt out very well. My favourite entry, other than Dr Iversen's traditional two, was that the Medical Research Council employs 106.5 million graduate staff. The head of personnel is right to worry about over-manning!

My favourite among all these four works, though, is Saur Verlag's *World Guide to Scientific Associations and Learned Societies*, one volume listing 22,000 organizations in all fields, science, technology and the other cultures; very catholic, I could have read it for hours. Here one encounters the American Underground-Space Association (not hollow-earthers, but engineers). And what about the Disinfected Mail Study Circle or the Japanese Correctional Association — it may just refer to the language but I am not sure. This book seemed to me to be truly valuable as a means to cultural insight, but most of the entries are in the language of their country of origin and are usually not translated. Also provided is an 8,000-entry index of the abbreviations for the associations, and then a breakdown by subject, another fascinating compilation. Under humanities, Britain rates only one entry along with Togo, Upper Volta, Iran and Israel, whereas France has 16 and the USSR none.

There is a fair overlap between this volume and the excellent *The World of Learning*, published by Europa, which lists 24,000 universities, museums and learned societies, for instance, and gives a fair amount of detail about each, managing to get in roughly 150,000 of the people associated with them; a new edition for 1984-5 comes out soon. But the strength of Saur's *World Guide* is its breadth — the placing of the large "official" organizations in a proper context of grass-roots societies. Science indeed is more than main-stream institutional research. □

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China: the promise and the problems

Gregory Blue

Science and Technology in China.

By Tong B. Tang.

Longman: 1984. Pp. 269. £45, \$85.

CHINA'S science and technology have developed rapidly since 1949, and in particular since the National Science Policy Conference of March 1978 which aimed at bringing China to advanced international levels by the year 2000. In this contribution to Longman's *Guide to World Science and Technology* series, Tong B. Tang (Deng Tang-bo) presents us with a concise, up-to-date account of the state of play in those fields of applied science and sophisticated-to-intermediate technology designated for priority development at that conference. This volume thus nicely complements the collaborative work, *Science in Contemporary China* edited by Leo A. Orleans (Stanford University Press, 1980), which surveyed the entire range of pure and applied sciences down to the end of the 1970s.

Following a general introductory analysis of science policy, organization and funding, the author proceeds in subsequent chapters to examine agricultural and environmental research, the earth sciences and mineral industries, energy science and technologies, as well as transportation and information technologies. For the chapter devoted to the biomedical sciences valuable contributions have been elicited from Hong Guo-Fan and Jin Neng-Ren, who write respectively about basic research and clinical practice. An interpretive sketch on science and social change in China by John Collier fills in the basic historical background in Chapter 2. The final chapter helpfully (though unusually) gives an *exposé* of the state of science and technology in Taiwan. One might also have liked to see included a similar chapter on Hong Kong (Xianggang, in the *pinyin* rendering of the standard pronunciation); but this is not a serious lack, since knowledge of work done there is already quite accessible.

The author takes due care to identify key institutes, to note which ones have been re-organized in recent years and to describe in some detail the work being done at each. Indication is given of those areas (such as microsurgery, earthquake prediction and satellite tracking and recovery) in which Chinese scientists have already attained advanced world levels. The author also points out that, having started from a very restricted industrial base before 1949, China has recently overtaken Japan as the world's foremost manufacturer of radios; she also now ranks as the fifth-largest producer of steel, although certain high-grade types still remain beyond her

competence. As in the Soviet Union, performance in the pure sciences is generally impressive. Like both the Soviet Union and Japan, however, China faces considerable problems in several areas in moving from basic research through the realm of research and development and into that of economic operationality. And Chinese technological capacity remains low in some crucial economic sectors. For example, in the coal industry 40% of pit faces are still unmechanized; and there are grave shortages in the transportation and energy sectors. Given present priorities, however, such circumstances may well be dramatically changed by the end of the century.

Chinese modernization in the early post-Mao years was predicated on a policy of massive and sometimes ill-considered technology transfer from the industrialized countries. In recent years this policy has been replaced by one of more selective transfer, often under the auspices of agencies of the United Nations. Emphasis seems to have swung towards importing a restricted number of examples of a particular technology and of using native expertise to appropriate it to Chinese conditions.

One supposes that, after a lag of several centuries, the task of bringing Chinese scientific and technological standards back up to international levels in most major fields will require a few generations more. Much progress has already been booked, however; in the light of China's human resources and history, it would be surprising if it were not to continue. At present it would seem ill-considered to keep oneself in the dark about the work of Chinese scientists, for there are few fields in which they have not made significant breakthroughs, as the volume under review shows.

Despite the diversity of its technical subject-matter, the presentation in *Science and Technology in China* is clear, concise and in general easily readable, though perhaps more attention at the sub-editing stage would have been in order. Conscientious internal cross-referencing together with efficient subject — and institutional — indexes make it a useful reference work. □

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● Other titles published in the *Longman Guide to World Science and Technology* series deal with the Middle East (by Ziauddin Sardar; reviewed in *Nature* 302, 359; 1983), Latin America (compiled by Latin American Newsletters) and, most recently, Japan (by Alun M. Anderson).

Later volumes will cover the United Kingdom, South-East Asia, the Indian Subcontinent, the United States, and Australasia, Antarctica and the Pacific.

Rolling and pitching in Russian

Vera Rich

Russian - English Translators' Dictionary: A Guide to Scientific and Technical Usage, 2nd Edn.
By Mikhail Zimmerman.
Wiley: 1984. Pp. 544. £31, \$59.95.
Dictionary of Scientific and Technical Terminology: English, German, French, Dutch, Russian.
Edited by A.S. Markov et al.
Martinus Nijhoff: 1984. Pp. 496.
Dfl. 90, \$34.50, £22.95.

DICTIONARIES, and technical dictionaries in particular, must surely be judged not only on the accuracy and contemporaneity of their contents, but also on how far they serve the needs of their intended users. These two volumes, very different in intention, differ too in their achievement of that aim.

Zimmerman's *Translators' Dictionary* has been a standby for the professional user since its first appearance in 1967. It is not a normal dictionary, listing not so much individual words and concepts, but the constructions linking them and the idiomatic turns of phrase which transform a piece of translationese into a readable text. Constructions are indicated by an example, usually a complete sentence, with the words in question printed in bold. Thus: "OTVERGAT' GIPOTEZU: By 1950, the tetranucleotide hypothesis **had been overthrown, (or ruled out, or rejected)**". This format is particularly helpful in coping with those Russian words which have a number of near-synonymous renderings. No less than seven examples are listed for "vrashchat'sya" in its various meanings of to rotate and to revolve, while for the derived phrase "vrashchat'sya vokrug osi" we have this splendid example: "Like an airplane, an insect can **roll around its longitudinal axis, pitch around a horizontal axis** perpendicular to its direction of flight, or **yaw around a vertical axis**".

A dictionary of this type cannot be employed without a good special-subject dictionary for the text in hand. In combination with such a work, however, Zimmerman's *Translators' Dictionary* has been proving its worth for almost 20 years. This new, updated edition will come as a boon to the younger generation of translators who, until now, have been obliged to work with library copies unless fortunate enough to inherit a copy from someone about to retire from translation.

In compiling their book, Markov and his colleagues have attempted to produce a "general purpose" dictionary, based on the vocabulary "pertaining to general study courses ... given in technical colleges irrespective of their specification".

Revealing as this is of the broad nature of such courses in Russian colleges (opening at random, one observes, pp. 62-63, that students have to be conversant with such varied concepts as cloud-chambers, co-factors, cold-working and collapsars), the result is a somewhat confusing conglomeration. Too many concepts are included for the book to be of more than partial use to anyone but a professional translator, who would, presumably, prefer a specialized dictionary for each topic.

A more serious complication stems from the fact that English is chosen as the main language (with cross-references in the other languages). This raises the difficulty of the differences between British and American spelling, which has here resulted in some odd inconsistencies. Both "center" and "centre" are listed, "center/centre of gravity" occurs with both spellings, but "center rest" and "centre of curvature" occur only once. Moreover, "center" is rendered as "centrum" in Dutch, while "centre" can be either "centrum" or "middelpunt"; and although "center of gravity" is "gravitatiecentrum", "centre of gravity" becomes, for some reason, "zwaartepunt". The compilers also have

a somewhat archaic view of English — was it really necessary to list "quicksilver"? — and seem to have a marked reluctance to list an adjective without a noun; thus, the adjective "mathematical" occurs six times, grouped with "expectation", "induction", "logic", "model", "pendulum" and "programming", although all the qualified nouns occur elsewhere (in some form) in the *Dictionary* and all are rendered by a straightforward noun + adjective construction (save for the German *Erwartungswert* which is allowed to drop its adjective).

The listing of complex phrases, though essential in a work such as Zimmerman's intended for the translator who has to pay attention to style, is unnecessarily cumbersome in a work meant for "scientists and engineers" whose need primarily is to read and understand professional material. It is particularly awkward in this book where the five-language format has led to crowding and illegibility which is further exacerbated by the generally cramped and unattractive layout. □

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Words, words, words

Michael Spencer

Concise Science Dictionary.

Edited by Alan Isaacs, John Daintith and Elizabeth Martin.
Oxford University Press: 1984. Pp. 758.
£12.95, \$22.50.

THE world's best-known lexicographer, in defining the word "dull" for his *Dictionary of the English Language*, gave as an example "To make dictionaries is dull work". One test of a dictionary is whether this shows through in the reading of it: would anyone choose to read it for pleasure and education, or only as a last resort when baffled and irritated by an unfamiliar word?

Much depends on the enthusiasm of the compiler, and it is probably inevitable that a committee job such as the present volume will be less fascinating (though more reliable) than the idiosyncratic brainchild of a single author. The editors have, however, achieved their well-defined aim of providing a handy and readable book of reference for school and first-year university students, and for laymen who want to know the meaning of the commoner scientific terms. A *Nature* reader bemused by a title such as "Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene" will at least be able to look up "transcription" and "oncogene", plus about one word in ten of the text of the article itself.

There are over 7,000 definitions (which could, incidentally, have been augmented

by omitting the spaces between entries); line drawings and tables are included where necessary, and there are useful appendices on units, fundamental constants and so on. One passes from "bark" to the "Barkhausen effect", and from "plate tectonics" to "platinum". The content is severely practical, so that although the quark is defined one looks in vain for the derivation of this intriguing word (it was, in fact, lifted from James Joyce's *Finnegans Wake*). The philosophy of science is not covered, so that Occam's razor and other concepts of incalculable value to the budding scientist are not mentioned; reduction in the chemical sense is defined, but reductionism is not.

It is interesting to note how some ordinary English words are used by specialists in different disciplines for quite different purposes: "accommodation", for instance, has three distinct definitions in animal physiology, botany and animal behaviour. One may quibble about the relative emphasis given to different entries — a dictionary offers unlimited scope for quibbling — but this probably again reflects the multi-author genesis of the work. While Ohm's law clearly deserves the paragraph devoted to it, one may question whether the ohmmeter (a meter with which you measure ohms) warrants an entry of the same length. On the whole, though, I think the book will successfully fill an obvious gap in the market, and will find its way into the Christmas stockings of young scientists of ages nine to ninety. □

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Pups and groundhogs

Alan Hendry

Dictionary of Ceramic Science and Engineering.

By Loran S. O'Bannon.

Plenum: 1984. Pp. 303. \$45, £42.75.

"CERAMIC science and engineering is one of the oldest yet one of the newest, fastest growing ... of man's endeavours". Dr O'Bannon begins his preface to the *Dictionary of Ceramic Science and Engineering* with this comment, and therein lies the problem for the etymologist. Ancient origins and the speed with which ceramic science is expanding and incorporating terms from associated disciplines make it impossible to provide a definitive compilation.

Dr O'Bannon recognizes these problems, and based on his wide experience of the subject he has made a brave attempt at providing a reference book to suit several tastes. In so doing, however, he has fallen between several stools. The *Dictionary* is not detailed enough for the professional ceramist and yet is too detailed for non-ceramists. Extensive sections are devoted

to rare earth compounds but the data are of limited value. The information on applications is useful but the representation used in giving chemical data is misleading; for example, the "silicon carbide composite" $\text{SiC} \cdot \text{Si}_3\text{N}_4$, molecular weight 180.28, would be taken to imply a chemical compound of one molecule SiC and one molecule Si_3N_4 , but which does not in fact exist.

The book is dedicated to promoting the standardization of terms in ceramic science and engineering and any attempt in this direction, particularly in a trans-Atlantic context, is to be commended. The bibliography provides a useful source from which to follow up the information contained in the text. Dr Johnson remarked that "making dictionaries is dull work", but omitted to mention that when made they are useful; in both senses this volume is no exception. Readers will no doubt find definitions with which they disagree, but sufficient reason for buying the book is that there is no better alternative available. □

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Business in science

W.F. Clocksin

Dictionary of Computers, Data Processing and Telecommunications.

By Jerry M. Rosenberg.

Wiley: 1984. Pp. 614. Hbk £29.95, \$29.95; pbk £14.95, \$14.95.

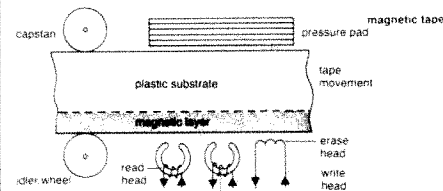
Dictionary of Computing.

Oxford University Press: 1984. Pp. 393. £15, \$25.

AT ONE and the same time computing is a thriving academic subject, a growth industry as well as a new hobby for many. Computer science, in which the fundamental principles of computation are studied, is a subject area of prime interest in universities and research centres. There has been explosive growth in the applications of computing to business problems, including commercial data processing, telecommunications and the technology of information handling. Such a rapidly expanding and diverse field calls for up-to-date reference books which bring together the principles with the practice.

The large *Dictionary of Computers, Data Processing and Telecommunications* is only partly a success. Containing more than 10,000 entries, it is directed mainly towards the commercial applications. Many entries are in fact extracts from other dictionaries and technical standards documents, while others are a mixture of marketing slogans and singular neologisms; I am uncertain whether it is justifiable

to confer respectability on such terms by documenting them in this way. Typographical errors are in evidence, and there are some spectacular blunders, such as equating the "holes" in semiconductor materials with the holes punched in paper cards. Together with other examples, this rein-



Picture "magnetic tape" and "read head"! (Reproduced from the *Cambridge Illustrated Thesaurus of Computer Science*, by Arthur Godman, published by Cambridge University Press price £4.95.)

forces my impression that the author does not know the territory well.

I prefer the *Dictionary of Computing*. With about 3,800 well-chosen entries it is much smaller than the previous volume, but it is superbly edited, with instructive entries and intelligent cross-referencing. Terms in computer science are strongly represented, but the commercial world of data processing is not ignored. Unlike Rosenberg's book, some entries include useful drawings and tables. This is a valuable and reliable reference for the student as well as the computing professional.

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In terms of computer graphics

Julian Gallop

Dictionary of Computer Graphics.

By John Vince.

Frances Pinter, London/Knowledge Industries Publishing, White Plains, New York: 1984. Pp. 132. £15, \$34.95.

ONE might expect a dictionary on a technical topic to contain rather formal definitions of a number of terms. In *Dictionary of Computer Graphics* John Vince does indeed follow the structure of alphabetically ordered entries, but he has chosen to unite them in a descriptive manner with generous use of figures. This gives a good introduction to the chosen topics, even if a few entries are rather longer than is usual in a dictionary. There is a slight danger in the simplicity that this encourages; for example, a statement made under "hidden-line removal" about a regular polyhedron can in fact be made with more generality about a convex polyhedron and would give more insight into the reasons for the statement. However the overall style does encourage the reader to explore the book.

My reservations are mainly about the organization of the *Dictionary* and the selection of terms for inclusion. There are cases where the specific can be consulted, but not the general; for instance one can look up Bézier curves (and patches) but not discover that B-spline is an alternative technique described in the book — if curve-fitting were an entry it could refer to both. In a field where terminology has not stabilized, and several terms are used for similar concepts, a dictionary has a duty to record them, because the reader will come across those terms elsewhere; thus picture plane is defined in the context of two-dimensional projections of three-dimensional scenes, but not projection plane or view plane, which are used in a well-regarded textbook.

Some terms used internally are not defined, while others are defined as a group; thus PAL, the method of encoding television signals used in Britain is described but cannot be looked up because it appears under NTSC which is the American system. The person learning to program some computer graphics applications will not find any mention of such commonly accepted terms as segment, viewport or pick. Possibly the book is not intended for such people.

The *Dictionary* could, then, have been more thorough and balanced. But it is attractively written and presented, and conveys an infectious enthusiasm for the subject. □

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Learning of the gods

P.W. Atkins

McGraw-Hill Encyclopedia of Chemistry.

Editor-in-chief Sybil P. Parker.

McGraw-Hill: 1984. Pp. 1,195. \$76.50, £48.50.

Van Nostrand Reinhold Encyclopedia of Chemistry, 4th Edn.

Editor-in-chief Douglas M. Considine.

Van Nostrand Reinhold: 1984. Pp. 1,082. \$89.50, £95.

HERMES Trismegistos, the Egyptian chemist (and god), was reputed to know everything and to have written it all down in 36,525 volumes. Latter-day Hermes must contend with publishers, pockets and profits, and must confine themselves to a 1,000 pages or so. But given that these two hefty volumes do summarize the present state of chemistry (a question we return to later), what impression would we take away from them about the range of the subject and its present achievements?

First, a few facts. The McGraw-Hill version (henceforth Hermes-M), cuts chemistry into about 800 pieces. The van Nostrand version (Hermes-N), slices more finely, and ends up with around 1,300. Hermes-M is extracted from the fifth edition of the *McGraw-Hill Encyclopedia of Science and Technology* (1982). Hermes-N claims to be fully revised and much enlarged from the earlier editions, with about 90% of the material being new. Both Hermes wear the hats of specialist contributors from around the world; but while M ascribes every entry to its author, N is much more coy and many pages sometimes pass before a name appears. I very much prefer M's approach in this respect: it is helpful to see an attitude subscribed by authority, especially if it conflicts with a prejudice. Moreover, if a point seems questionable, it is useful to know that the apparent cross-support from another entry is written by another hand.

Quite different impressions of chemistry are obtained at first glance. Hermes-N is conventional in appearance with a somewhat muddy page; the line drawings are amateurish and poorly labelled. Hermes-M, on the other hand, is much more striking in appearance, with a page that looks inviting, neatly tinted boxes displaying structures, and well-drawn illustrations. Both have quite good, but not perfect indexes, which are essential in works as big as these. Here N wins hands-

down, with around twice as many entries as M. The smaller count reflects M's lower density of cross-referencing; for example, "orbital" does not appear as such in M, but has to be sought under "molecular orbital", which is not quite helpful enough, at least to a non-specialist.

It is once we move beyond first impressions that we get strikingly different visions of the present state of chemistry. From N it seems that a chemist is one who knows about sphene, spinel, spodumene and spray drying (to take a page at random). A page more typical of M contains second-order transition and SIMS. Hermes-M knows not of spodumene and spinel. Hermes-N is free of SIMS.

Hermes-N is the traditional fellow, the god of foremen, plant managers, geologists and agriculturalists. N is largely the god of the technological chemist (who might understand his old-fashioned units). While N gives some helpful synoptic surveys of large fields (antibiotics for example), which are missing from M, there

are some bizarre judgements. Why no mention, for instance, of NMR, probably the single most important physical technique available to chemists? Why six pages with do-it-yourself style diagrams on the fabrication of optical fibres?

Hermes-M, on the other hand, is a much more academic god. He seems to know little of traditional chemistry but a lot about spectroscopy, vinylology and oscillating reactions. No nut-shell carbon for him.

For information about traditional chemistry, and particularly chemistry with a technological tinge, then Hermes-N is appropriate but a bit old-fashioned. For information about modern aspects of chemistry, with an up-to-date view of its current achievements, then Hermes-M is undoubtedly the better source (and, at the price, the better value). □

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Eminent lives

Christopher Spencer

Biographical Dictionary of Psychology.

By Leonard Zusne.

Aldwych Press, 3 Henrietta St, London: 1984. Pp.563. £47.50, \$49.95.

ABRAHAM, Karl (German psychoanalyst) to Zwaardemaker, Hendrick (Dutch otolaryngologist), but this is far from being just a dictionary of psychologists. Zusne has provided an excellent source of reference to the intellectual history of psychology: 544 brief biographies which, taken together, trace the development of ideas from Thales, Pythagoras and Heraclites, through the mediaeval scholastics and Arab philosophers, to the origins of modern science and the emergence of psychology as a distinct discipline.

Each entry has a common format: place and exact dates of birth and death; highest degree awarded; positions held; then several paragraphs describing and evaluating the work of the person concerned and its significance for psychology; and finally a bibliography of fuller biographies of the individual.

One can thus use the book as an historical encyclopaedia or browse with profit through the excellently-summarized biographies: Zusne shows that brief entries can be succinct without trivializing the subject matter. The volume will serve well as a sourcebook for amateurs in the history of science, especially those interested in the cross-influences between disciplines, and includes an appendix which lists the nineteenth- and twentieth-century contributors to psychology by their principal university or institution. This appendix dramatically illustrates the Germanic domination of

nineteenth-century science, and the shift to American pre-eminence by the early twentieth century. Indeed, many individual biographical entries show that there was a movement of persons as well as ideas: Jastrow, Rapoport, Razran, Reich, Spitz and many others were born and educated in central and eastern Europe, but spent much of their working life in the United States.

Another appendix is perhaps of lesser value: Zusne takes up 16 pages with a table of "relative eminence", a ranking of all the contributors to psychology according to a count of pages devoted to each individual in 16 histories of psychology. Thus, in this table, Maslow beats by a short head St Albertus Magnus, who in turn ranks one place ahead of Kraepelin.

The only serious limitation of the book stems from Zusne's understandable reluctance to adjudicate between the relative claims of the living for inclusion in the *Dictionary*, and his consequent limitation of entries to the distinguished deceased. This imposes a curious selectivity of coverage of the subject. Thus, for example, Berlyne, Olds and Lenneberg, born in the 1920s, are eligible for inclusion because of their relatively early deaths; whereas many equally eminent figures of twentieth-century psychology are still happily living, and thus ineligible.

This limitation on one side, the *Dictionary* is a welcome reference work. One final thought for our own over-productive age that remains with me, after several pleasurable hours of browsing, is that many of these biographies demonstrate that impact does not necessarily require a large output. □

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● Also newly published by McGraw-Hill is the *McGraw-Hill Dictionary of Chemistry*, containing terms and definitions selected from the third (1984) edition of their *Dictionary of Scientific and Technical Terms*. The chemical dictionary includes more than 9000 terms with emphasis on the vocabulary of theoretical and applied chemistry, and costs \$44.95, £28.50.

Pieces of mind: more for the clinician than the academic

Stuart Sutherland

Longman Dictionary of Psychology and Psychiatry.

Edited by Robert M. Goldenson and Walter D. Glanze.

Longman: 1984. Pp. 816.
£29.50, \$39.95.

THERE is a real need for an up-to-date dictionary of psychology; as far as I know, none has been published since 1978. Despite the fanfare in the blurb ("21,164 entries or headwords, twice to five times the number of other dictionaries in these fields"), the *Longman Dictionary of Psychology and Psychiatry* fails to fill this gap.

The psychological entries contain many errors and omissions. The latter include many terms in common use, such as "two-process learning theory", "episodic memory", "iconic storage" and — less common, but still important — "anorthoscope". Students frequently confuse "focussing" with "fixating", but this book will not help them: "fixating" is not the "focussing of both eyes on a single object", it is positioning the eyes in such a way that the same object falls at the centre of each fovea (the same error recurs elsewhere in the *Dictionary*). The definition of "receptive field" would have been correct fifty years ago, but does not capture the way the expression is most often used today. Many Pavlovian terms are too loosely defined — "Pavlovian inhibition" is not the "suppression of a response", it is a *process* that may lead to the suppression of a response; the unconditioned stimulus follows the conditioned one, it is not merely paired with it and so on.

A half-hearted attempt is made to cover some of the fields that are now relevant to psychology, for example linguistics, but oddly there appear to be almost no terms from computing science or artificial intelligence: the definition of "heuristic" is grossly misleading since its technical sense is omitted.

The *Dictionary* appears to do better with psychiatric terms, of which it includes many more than the latest edition of Oxford University Press's *Psychiatric Dictionary* (1981); hence, it may be of more use to psychiatrists than to psychologists. But when confronted with this weighty volume, members of both professions may well wish that progress in their respective fields had been commensurate with the amount of jargon they have generated. □

Stuart Sutherland is Director of the Centre for Research on Perception and Cognition at the University of Sussex.

Environmental issues

Timothy O'Riordan

Dictionary of the Environment, 2nd Edn.

By Michael Allaby.

Macmillan, London: 1984. Pp.529. Hbk £25; pbk £7.95.

Directory for the Environment: Organisations in Britain and Ireland 1984-5.

Edited by Michael J.C. Barker.

Routledge & Kegan Paul: 1984. Pp.281.
Pbk £8.95, \$19.95.

PRODUCING a dictionary must be a labour of love. The intellectual rewards are relatively small and the scope for criticism over omissions or nuances of meaning is considerable. Michael Allaby's second edition of *Dictionary of the Environment* comes six years after his first. The contents are much fuller and he has benefitted from the advice of a small company of distinguished friends. The entries are mostly very short, covering clearly and competently all the basic physical, chemical, biological and geographical phenomena. The book's conciseness is a great asset: some 6,000 entries are crowded into 529 pages. However some entries are superfluous — or at least appear so because of the apparent determination to be brief. For example "Environment, Department of" is described as "Department of the Environment" — no mention as to country, or function or date of birth. Similarly EPA is merely described as "Environmental Protection Agency". This is hardly helpful, and a relatively modest expansion of information would have paid dividends.

There are also some curious omissions. There is no entry for environmentalism or environmentalist, nor is there anything under acid rain or acid deposition. There is an entry under environmental quality standards (an American term now embraced by the European Commission), but nothing for environmental quality objectives, a concept much protected by British regulatory authorities. In similar vein, the entry under "best practicable means" only connects it to air pollution control when it now has much wider application. The notorious phrase "as low as reasonably achievable", now in such dispute in the wake of events at Sellafield, does not appear at all. This is a dictionary of specifics rather than concepts.

Michael Barker's *Directory* is altogether a more worthy book, even though it was probably easier to collate than Allaby's *Dictionary*. Barker has sought to compile a list of all official and voluntary organizations which have something to do with environmental management and environmental protection. He appears to have sought his information with care but within a limited budget: inevitably some of the entries are piecemeal and inaccurate. As this is to be the first of a series, greater

comprehensiveness and accuracy should be attained later on.

The entries are listed in alphabetical order. Each provides a name, address, contact phone number and a brief description of activities together with a list of relevant publications. There is a good subject index which allows the reader to look up the variety of organizations interested in a particular theme. For example there are 34 entries under "energy conservation" and 67 under "nuclear power". Maybe next time Barker will delve into the *Civil Service Yearbook* to seek out the sub-compartments of government departments responsible for particular aspects of environmental matters.

Nevertheless this is a valiant and valuable production. Barker has uncovered all kinds of interesting groups. What about HOOVES, which aims to make roads a safer place for horses and to encourage the provision of livery stables and "parking" places for horses in cities? Or Green Deserts, which promotes and participates in desert reclamation schemes and seeks to bring the facts about desert reclamation to the attention of the general public? Students and researchers should take note: many of the organizations listed provide information upon request and they are often well qualified and motivated to do so. Here is an excellent reference book. □

Timothy O'Riordan is Professor of Environmental Sciences at the University of East Anglia.

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Green and pleasant

Richard Mabey

The Macmillan Guide to Britain's Nature Reserves.

Edited by Robert Boote.

Macmillan, London: 1984. Pp. 717. £30.

RSPB Nature Reserves.

Edited by Nicholas Hammond.

RSPB/Croom Helm: 1984. Pp. 189. £9.95.

WITH the centenary of the first nature reserve (Breydon Water in Norfolk) and the 900th anniversary of the Domesday Book both only a few years away, it is an apt moment to produce an inventory of Britain's protected acres. Macmillan have sidestepped ideological wrangles about whether reserves exist principally for wildlife or for the human beings who created them by publishing a monumental guide that is both reverent of its raw material and thoroughly populist in approach. The basis for inclusion of a site, it states, "is that members of the public should have access either by common law rights, or by membership of a club or trust". There is easy public access inside the book, too, thanks to a clear and forthright design, simple, foolproof keys (with map reference, acreage, ownership, best time of the year to visit) and vivid descriptive accounts of the natural features of each site that on the whole avoid scientific jargon.

And what a feast it all adds up to! Seven hundred pages containing entries on some 2,000 reserves, ranging across Britain from Kyemel Crease in Cornwall, a tiny patch of scrub woodland developed over 121 abandoned cliff-top fields, to the puffin-stacked cliffs of Hermaness National Nature Reserve in Shetland. Every imaginable kind of sanctuary is featured: flooded brickpits, old commons, municipal country parks, water authority reservoirs, the occasional free-enterprise

private wood and whole National Parks. The potential users of the book are just as various. For apprentice twitchers it will be an invaluable crib, thanks to its species index; for holidaymakers it would work as a rewarding alternative tourist guide. (Try it out on unpromising Notts, and read about the splendid gravel pits called Attenborough!)

It would be possible to see this book, only slightly cynically, as *The Good Habitat Guide*, and have a melancholy vision in which spontaneous encounters with nature are superseded by something approaching reserve-crawling. At £30 a copy though, I rather doubt it. In my local library they have placed it on the table between the tourist leaflets and the telephone directories, and that seems to me an accurate and rather complimentary assessment of its social role.

Those who prefer their guidebooks to provide ecological background material as well as information, can turn to the Royal Society for the Protection of Birds' account of its own holdings. There are less reserves covered here (some 90, occupying over 100,000 acres) and the book is oddly evasive about access arrangements, but as a bonus there are a number of concise and thoughtful essays on the history and management of different kinds of reserve habitats.

Although I personally find the necessity for nature reserves a sad commentary on our mistreatment of the natural world, I warmed to these two books. It is, after all, better that the last oases are preserved than not. And it would be hard not to be lifted by the images that shine through both guides — of natural landscapes the immense variety of which can still be experienced, and of the people whose hard work and devout attentions have saved their remaining fragments. □

Richard Mabey is a member of the Council of the Nature Conservancy Council, and a writer on countryside matters and landscape history.

Poisoned principles

J. D. Phillipson

Poisonous Plants in Britain and Their Effects on Animals and Man.

By Marion R. Cooper and Anthony W. Johnson.

Her Majesty's Stationery Office: 1984. Pp. 305. £12.95.

A Colour Atlas of Poisonous Plants.

By Dietrich Frohne and Hans Jürgen Pfänder. Translated by Norman Grainger Bisset.

Wolfe Scientific, London/Sheridan House, White Plains, New York: 1984. Pp. 291. £30, \$38.

WHEN two books on the same topic emerge at about the same time, it might be thought that the reviewer should be able to assess which one is the better buy. In this case it is not possible because these two books in fact complement one another rather than compete.

The MAF Bulletin 161, *British Poisonous Plants*, was last reprinted in 1979 and was in need of a complete revision. M. R. Cooper and A. W. Johnson have done just that, bringing together the botanical and veterinary aspects of poisonous plants. The most common cases of animal poisoning are due to bracken, ragwort, brassica, yew, oak and the umbelliferous plants. Treatment is briefly discussed with the sensible caveat that "the doctor and veterinary surgeon . . . should be consulted immediately". A section on poisonous principles describes various alkaloids, glycosides, nitrites, oxalates, photosensitizers, proteins, peptides, amino acids and tannins, but no structural formulae are provided for the chemically minded reader.

The book is then divided into sections on fungi, pteridophytes and the flowering plants, the species being listed within their families which are in alphabetical order instead of the former taxonomic order. Over half of the book is devoted to the families, ranging from Alliaceae to Umbelliferae, and each plant is discussed under a series of headings such as poisonous principles, poisoning in animals, human poisoning and treatment. There are some colour prints but these are relatively few, the first one not appearing until p.94, and the photographs are of plants in their natural habitat so that they are not necessarily easily recognized. Each section is supplied with a liberal number of references and there is a good general index.

How does the volume by Frohne and Pfänder differ? First, and most obviously, it is beautifully presented with much better, clear colour photographs usually taken against a light background. Each plant, again listed in alphabetical order of families, is illustrated, and in many cases there is a photomicrograph or microscopical drawing of diagnostic features



From *The Macmillan Guide to Britain's Nature Reserves*.

Remnant beauty — Tennyson Down and the first of the Needles, off Britain's southern coast.

which aid identification. Structural formulae abound and there is a clearly marked section on treatment of poisoning for each plant.

This volume is a practical one, designed to help quick and easy identification of poisonous plants and, except for one page, is concerned with human not animal poisoning. Fungi are considered to be outside the scope of the book but a number of ornamental "non-British" plants such as *Dieffenbachia* are included, an important practical addition.

Should we be concerned about poisonous plants when only two deaths have been reported between 1962 and 1978 in Britain? Clearly the answer must be yes

because 20,000 enquiries were made at the National Poisons Information Service centres, and there were up to 60,000 plant-poisoning cases of children in Germany. Furthermore, there is not just concern over yew and laburnum but also increased public interest in herbal medicines. Both books take careful stock of such medical plants as comfrey and mistletoe, making balanced judgements on the pros and cons of taking such medication, and are, overall, two lovely productions which were a pleasure to review. □

J.D. Phillipson is Professor of Pharmacognosy at The School of Pharmacy, University of London.

Talk of plants

C.A. Stace

The Penguin Dictionary of Botany.
Edited by Stephen Blackmore and Elizabeth Toothill.

Penguin: 1984. Pp. 390. Hbk £12.95; pbk £3.95. In the United States The Facts on File Dictionary of Botany (Facts on File, hbk \$21.95).

Longman Illustrated Dictionary of Botany.

By Andrew Sugden.

Longman: 1984. Pp. 192. £3.95, \$7.95.

SPECIALIZED jargon is one of the more daunting obstacles to the understanding of a technical subject, and that of plant biology poses particularly severe problems — many separate disciplines are involved, and the subject has been accumulating technical terms for over 2,000 years. There would be little use to working scientists or students for an encyclopaedic listing of every term that has ever been coined. Hence the main difficulty for the compilers of desk-top dictionaries is to find a middle way; their products must be small enough to be manageable yet large enough to be informative. These two books both strike the right balance.

The Penguin Dictionary of Botany (hereafter *Penguin*) contains "over 3,000" entries, compiled by ten contributors under a general and a consultant editor, while *Longman's* offering has "over 1,200" compiled by a single author. The audience for the two books differs, but with some overlap. *Penguin* is quite sophisticated and will appeal widely to sixth formers, undergraduates (especially) and to the professional plant biologist. *Longman* is more elementary and will find its greatest use at the school-university interface.

The main feature of *Longman* is the large number of colour illustrations (averaging more than one per page), and the grouping of terms into 24 subject sections, necessitating a separate index at the end of the book. *Penguin*, by contrast, is entirely alphabetical and carries relatively few

illustrations, most of which are charts and formulae, and some of which are rather unnecessary (over half a page for chlorophyll lamellae, for example). Important entries are given a more extended treatment; there is, for instance, almost an entire page devoted to the chloroplast, excluding the illustration. In each book the cross-referencing is excellent.

Both the dictionaries seek to cover more or less the whole range of "botany", including bacteria and viruses, except that agriculture and horticulture as well as plant biotechnology seem to be omitted by *Longman*. The latter scores, however, by its inclusion of useful appendices on Greek and Latin prefixes and SI units. Both books would have profited from more appendices on topics such as the periodic table, a system of classification and so on.

Criticisms of omissions must be very subjective, but *Longman* seems to include rather too much classical terminology (e.g. polyadelphous) at the expense of more modern aspects (e.g. plasmid). Errors of fact, however, are less disputable, and I was disappointed to see a major error perpetrated on p. 47 of *Longman*. Here crossing over is shown (unequivocally in multicolour) to occur at the position of a chiasma, that is genetic crossing over is said not to occur until after chiasma formation, whereas the reverse has been known to be the case for over half a century. Such misinformation is potentially harmful in an often-misunderstood though essentially elementary topic. Seeking reassurance, I turned to the corresponding entries in *Penguin*, and found, by implication, the same mistake. These are certainly points requiring urgent rectification at the first opportunity.

How successful these dictionaries will prove will become clear only after trial periods with the intended users. But my general impression is that much thought and attention have gone into both, and that both should (and deserve to) become well used. □

C.A. Stace is Reader in Plant Taxonomy and Head of the Department of Botany at the University of Leicester.

Order in mammals

Malcolm C. McKenna

Orders and Families of Recent Mammals of the World.

Edited by S. Anderson and J. K. Jones, Jr.
Wiley: 1984. Pp. 686. £63.55, \$54.95.

CHARACTERIZING and keeping track of the taxonomic hierarchy of thousands of genera of mammals distributed among about 30 orders is no easy task, as a succession of classifiers since Linnaeus has found out. In 1967 the editors of the present volume published *Recent Mammals of the World*; now they have updated and retitled it. As before, about 20 highly qualified contributors, some inherited from the first project, cover all living mammalian orders and the constituent families in a reasonably uniform style but with a range of originality.

Each still-living order is given a diagnosis, followed by short synopses of current distribution, geological range and a list of constituent families; there is also a section of remarks in each case. Then the format is followed again at the family level. Unfortunately many of the diagnoses contain characters that are merely descriptive, not diagnostic, but they are still useful.

A separate, well-written and generally succinct account is given of fossil mammals, including groups without living members. The known diversity of extinct mammals is increasing rapidly, mainly through the efforts of palaeontologists using screen-washing methods. This is especially true of small rodents and insectivores, but also applies to other orders. Since the 1967 compilation the number of known fossil genera of rodents alone has doubled, surpassing for the first time the number of living genera of rodents.

Among the accounts of living mammals, five chapters on rodents, one on bats, and one on monotremes and marsupials are outstanding, reflecting the enormous amount of recent work in those areas of mammalogy. The original scholarship displayed means that purchase of the book will be a necessity for many palaeontologists.

The illustrations consist mainly of distribution maps, but a number of figures of animals show enough anatomical detail to be helpful in identification (although some have been reduced excessively). Most of the anatomical illustrations are of the skeleton or skull, but dental patterns are not emphasized. An especially valuable feature of the book is the bibliography; the references occupy almost a hundred pages, and many of them are new since the 1967 edition. □

Malcolm C. McKenna is Frick Curator in the Department of Vertebrate Paleontology, American Museum of Natural History, New York.

Primate profiles

R.D. Martin

Primates of the World: Distribution, Abundance, and Conservation.

By Jaclyn H. Wolfheim.

University of Washington Press/
Harwood Academic: 1984. Pp. 832.
\$46 (US), \$72 (Britain and Europe).

WITH the exception of the *Red Data Book (Mammalia)*, a publication only partly devoted to primates and limited to the most basic information, there is no standard reference work regarding the conservation status of primates. In *Primates of the World*, Jaclyn Wolfheim has set out to remedy that situation.

In the book, a vast amount of literature is covered (there are over 1,000 references) and the available information is brought together into a well-designed framework. The text deals with the primates on a species-by-species basis and for each species provides information on geographical distribution, population parameters, habitat conditions, factors reported to affect wild populations, and conservation action under way. The profile for each species is accompanied by a distribution map and (where data exist) by a table summarizing population parameters. The distribution maps, which generally indicate *maximum possible* areas of present occurrence, have been carefully prepared and provide reasonably accurate guidelines. In this and other respects, Wolfheim made a concerted effort to ensure accuracy by incorporating results from questionnaires circulated to 226 field workers.

The reference value of *Primates of the World* has been enhanced in a number of ways. Most particularly, it is commendable that the author took pains to use a broadly comprehensible and acceptable scheme of classification; only a conservative scheme, such as the one adopted here, is likely to be readily understandable to the many different groups of people who are likely to use the book. Secondly, the species profiles are followed by a 20-page discussion that identifies a number of general principles, and by seven summary tables (one as an appendix) listing quantitative information across species. Two especially useful tables are one listing "major determinants of population status" (including an estimate of the maximal geographical range for each species in square kilometres) and another relating scores for these determinants to overall conservation status. The quantitative data provided have not been analysed by Wolfheim herself, but should provide at least a provisional basis for mathematical modelling in primate conservation programmes.

However the book does have its limitations. Perhaps unavoidably, it is already quite out of date, coverage of the literature

effectively ending at 1978 (all the references for 1979 and 1980 seem to stem from two edited works). Further, there are no descriptions or illustrations of individual species; hence anyone not already familiar with living primates will need a back-up reference source. It is also a pity that Table 190 gives body weights for only just over half of the primate species, and even then cites maximal body weights largely derived from captive animals. Finally, some of the information (for instance on primate importation and on the associated

legislation) is specifically relevant to the United States only, rather than being genuinely international.

But, overall, Jaclyn Wolfheim has done an excellent job. With one-third of the present total of about 150 primate species already listed as endangered, rare or threatened by the International Union for the Conservation of Nature, a source-book such as this was urgently needed. □

R.D. Martin is Professor of Physical Anthropology at University College London.

A view of life

A.J. Cain

A Synoptic Classification of Living Organisms.

Edited by R.S.K. Barnes.

Blackwell Scientific/ Sinauer: 1984. Pp. 273. Pbk £7.50, \$11.50.

DR BARNES has organized a most useful little book, giving a quick view of the diversity of living things, and designed as "the sort of dictionary/mini-encyclopaedia of organismal classification and diversity that all professional, amateur and student biologists could have on their desks". As he remarks, "student biologists are taught less and less of the diversity of life". It is hoped that this book will do something to remedy the defect; yet since it goes only to orders, and there is a single illustration for each phylum, it can do so mainly by sending its readers to more widely-illustrated or detailed texts. As such, it will be valuable.

The classification is a consensus, and avoids some of the wild splitting of recent texts. Viruses are excluded as not coming within the definition of living things, and

only groups with *some* living representatives are admitted — for example, the Merostomata are in, because of the horseshoe crabs, but the trilobites are not; the Chelonia, Rhynchocephalia, Squamata and Crocodilia are in but the various dinosaurs, ichthyosaurs and plesiosaurs are not. Perhaps a later edition can include them.

The Introduction is so brief as to be misleading in one particular: "Taxonomic categories of whatever level are arbitrary, man-made distinctions imposed on a continuum of natural variation" is a dangerous half-truth. It is *not* open to anyone to include human beings, *Drosophila* and snails in a single group. As G.G. Simpson has stressed, taxonomic categories are arbitrary by exclusion, not arbitrary by inclusion. And there isn't a continuum — indeed the definition of phylum status provided in the book is based on gaps.

No one will be entirely satisfied by the classification given here, but the five collaborators have done a good job. May I assist by pointing out that the Marsupialia have been divided into several orders for some time now? □

A.J. Cain is Derby Professor of Zoology at the University of Liverpool.



Tadarida pumila, a molossid bat, drawn by Jonathan Kingdon. The illustration is reproduced from the new paperback edition of Vol. IIA (*Insectivores and Bats*) of Kingdon's magnificent series of books, *East African Mammals: An Atlas of Evolution in Africa*, originally published by Academic Press. Volumes I, IIA and IIB are now available in large-format paperback, published by The University of Chicago Press. Price is \$25, £23 for each volume.

Feel for vertebrates

D.R. Newth

Longman Illustrated Animal Encyclopedia.

Edited by Philip Whitfield.

Longman: 1984. Pp. 260. £25. In the United States the Macmillan Illustrated Animal Encyclopedia (Macmillan, \$35).

DO NOT be deceived by the title's implied claim that this book surveys the whole animal kingdom, and still less by the dust-cover which calls it a "visual who's who of all the world's creatures". Instead, more modestly, it treats of some 2,000 species of living vertebrate animal. Each is granted a reproduction, in colour, of a painting and a short (80–300-word) non-technical description. The aim has been to include at least one species from each tetrapod family and at least one from each order of the fishes. Some higher taxa are briefly described and mammals, birds, reptiles, amphibians and fishes are each introduced by a short general account.

The pictures are certainly both vivid and decorative, but they are not always

sufficiently detailed or accurate to match good colour photographs (although, of course, good photographs would not always be easy to obtain). Since they are rationed at one to each species there is no place for larval or juvenile forms, and where sexual dimorphism is marked the more flamboyant sex is chosen for show. The textual material is necessarily basic, but has lacked critical revision to eliminate minor errors and other lapses from grace. Of the latter, the heading given to the section on reptiles, which boldly proclaims them to be "survivors from a prehistoric age", seems typical. It is true, but true also of every other class of living animal.

Its defects apart, it is unlikely that this book will be of value to students of vertebrate zoology. On the other hand it may well attract and instruct non-scientists and give them a feel for the range of size, shape, coloration and lifestyle of the vertebrates and some idea of their relationships. This, presumably, was the intention of the publishers, but it is still difficult to imagine whose coffee table the book is designed to grace. □

D.R. Newth was formerly Regius Professor of Zoology at the University of Glasgow.

Down under standard

G.B. Corbet

The Complete Book of Australian Mammals.

Edited by Ronald Strahan.

Angus & Robertson: 1984. Pp. 529. £25.

THE past 20 years have witnessed a revolution in the application of high-quality colour printing to natural history publishing. In that same period the production of authoritative syntheses of biological data on major taxonomic groups and faunal regions has achieved increasingly high standards. But with some exceptions, the economics of publishing have usually prevented these advances from coming together. Writers and editors of scholarly works are commonly frustrated by the impracticality of including colour illustrations, even when these would enormously enhance the information content, whilst popular works with adequate, and often superb, colour illustration rarely carry a text that meets the standards required of a scientific source book.

The exceptions have mostly dealt with subjects such as molluscs and butterflies, where the ease of photographing preserved specimens and the popular interest have combined to make good illustrated handbooks feasible. The elusiveness of most mammals makes them a much more difficult subject for such treatment, and so *The Complete Book of Australian Mammals* is something of a pioneer. It is based upon a

National Photographic Index of Australian Wildlife established by the Australian Museum, Sydney, and includes colour photographs of living animals of all but 22 of the 273 species included. Considering the rarity and elusiveness of many Australian mammals, marsupials and placentals, this is quite an achievement.

Although the convergence and parallelism shown by the Australian marsupials in relation to placental mammals elsewhere are a standard ingredient of evolutionary teaching, the illustration of almost all the 129 species in one work enables this phenomenon to be appreciated and indeed studied in a way that has not hitherto been possible.

The text is the work of 107 different authors with first-hand research experience, and is up-to-date and authoritative, summarizing habitat, ecology, reproduction and behaviour. The reference value is greatly enhanced by a clear distribution map for each species. Although facts are not individually documented with regard to source, some bibliographical references are given for each species, providing at least an entry into the literature.

While it falls short of being a really comprehensive illustrated handbook — it does not, for example, cover critical identification requiring internal characters — this book nevertheless sets a standard that has not yet been matched in areas such as Europe and North America where the mammal fauna is much better known. □

G.B. Corbet is in the Department of Central Services at the British Museum (Natural History), London.

Everyone's stunning animals

Carolyn M. Crockett

The Encyclopedia of Mammals.

Edited by David Macdonald.

George Allen & Unwin/Facts on

File: 1984. Pp. 895. In Britain published in two volumes, £25 each. In the United States as a single volume, \$45.

HERE is a volume whose illustrations and photographs are a pleasure to peruse. What makes it an especially worthwhile purchase, however, is the authoritative and well-written text. David Macdonald has orchestrated a lively collection of articles, assembling contributions from 180 experts from around the world; the result will appeal to anyone interested in animals — the lay public, students and their teachers, and professional zoologists.

The book describes the appearance, diet, social behaviour, anatomical characteristics and peculiarities of all mammal groups. For some, illustrations and descriptions of extinct ancestors are also included. The entries are all short, ranging from a small box on a specific topic to several pages of description of a mammal group, usually at the family or subfamily level. This makes ideal reading with morning coffee or afternoon tea, since the individual passages hang together and can be read in just a few minutes.

The book begins with a dozen or so pages describing mammals in general, summarizing their evolution, characteristics and taxonomy. The remainder is divided into accounts of each order and concludes with a bibliography, a glossary of nearly 500 terms that might not be familiar to the lay reader, and an index. For most orders, a complete listing and short description of each species is given in a section of the main text. A few orders, such as the Rodentia, Chiroptera and Marsupialia, are described down to the genus or tribe level, while a complete listing of species is presented in an appendix.

To give an indication of the richness of this volume, let me summarize the section on the Capuchin-like monkeys (Cebidae). It begins with a box containing a distribution map and a silhouette scale-drawing comparing the size of a man with the largest and the smallest members of this monkey group. The text includes a general description of the Cebids, their adaptations (such as prehensile tails for the larger species) and their social organization. A beautiful colour drawing (one of many excellent ones by Priscilla Barrett) accurately depicts nine species in various postures; by showing different — yet typical — behaviours, this and the other plates provide more information than do most field guides. Next comes a more traditional description of each species, including common name,

scientific name, pelage, distribution and so on, and finally four "topical" accounts of one to two pages each — here, exploration and play in Squirrel monkeys; why Night monkeys are nocturnal; the family life of Titi monkeys; and the energetic constraints imposed on Howler monkeys by their relatively unnutritious diet. Photographs are interspersed throughout.

As an amateur photographer and illustrator, I feel compelled to emphasize the sheer beauty and artistry of the layout and illustrations. Many of the photographs are stunning, portraying the very essence of an animal's life. One of the most dramatic depicts a herd of wildebeest plummeting into a swirling river.

There are really only two drawbacks to the book, both of them minor. First, no specific references are given (though each entry is initialled and in many cases the author's publications could be consulted). Second, while several passages, especially the "topical" ones, discuss controversial matters, in some instances no indication is given that this is the case; the problem then arises of preliminary conclusions being perpetuated as truths. But it is yet another merit of this splendid work that it corrects a far greater number of myths than it creates.

Carolyn M. Crockett is a Research Associate in the Departments of Anthropology and Psychology at the University of Washington, Seattle, and at the Smithsonian Institution, Washington, DC.

Mammoth appeal

Brian Bertram

The Mammals of the Southern African Subregion.

By Reay H.N. Smithers.

University of Pretoria: 1983. Pp. 736. Distributed by Struik Book Distributors, PO Box 1144, Cape Town 8000, South Africa. £53, \$70.

PRODUCING this major reference work on southern African mammals is described in the foreword as "a mammoth undertaking". It is a very apt description, for mammoths are also enormous, still too little known, fascinating in detail and just a bit dated in appearance.

Reay Smithers has managed during a relatively short gestation period of only about four years to assemble and present a vast amount of information. The three-quarters of a million words cover 340 species of mammal, and there are tables, illustrations and distribution maps as well. It is formidable — and remarkable that the book is still readable too.

The "Southern African Subregion" includes South Africa, Namibia, Botswana, Zimbabwe south of the Zambezi River and the Prince Edward Islands (situated 900 miles south of the con-

tinents), as well as the adjacent oceans. The latter provide an excellent excuse for a useful section on marine mammals — the whale and seal orders. Each species entry is divided between several subheadings: colloquial name; taxonomic notes (covering subspecies and disagreements over classification); description (including, at last, valuable tables giving data on body sizes and weights); distribution; habitat; habits (an inadequate term for the wealth of behaviour and ecology described); food reproduction; and skull (with dental formula).

The possible disadvantage of a standard format such as this is that sparse or trivial data might be included for some species (because there is a heading for them) and major topics omitted for better documented species. Fortunately Smithers has not felt constrained by the format: he includes introductory and general sections, as well as topics of particular interest (such as fur seal harvesting, and the multimammate mouse and human diseases), and he makes quite clear where adequate information is lacking. The gaps are fast being filled in, and what would have been only a skeleton of a mammoth 20 years ago is becoming beautifully padded out, thanks to the high quality of field work carried out and published over the past two decades or so.

The book's publication in South Africa in 1983, and its recent availability worldwide, make clear the extent of the takeover by southern Africa as the centre for exciting work on wild African mammals. Fifteen years ago, East Africa with its wildlife research groups particularly in Serengeti, Gombe, Nairobi, Tsavo and Mweya, was leading Africa if not the world in large-vertebrate field studies. For many reasons — political, financial and personal — the East African centres have become reduced. At the same time, the value of such research institutes has been amply demonstrated by the success of the Mammal Research Institute in Pretoria, which has both sired this excellent volume and provided much of the field data on which it is so soundly based. Fortunately, the whole of Africa, and indeed the whole mammal world will benefit. □

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● One of the best-selling publications issued by the British Library's Science Reference Library in London, *Guide to Government Department and Other Libraries*, has recently appeared in a new edition (the twenty-sixth). The entries cover not only sources of information within Government departments, but a selection of other specialist libraries and include many dealing with science and technology.

The *Guide* is available from: External Relations and Liaison Section, Science Reference Library, 25 Southampton Buildings, London WC2A 1AW, UK. Price is £10 (£11.28 for overseas orders); cheques should be made payable to The British Library.

Up in the air over the nation

John Andrews

A Field Guide to the Birds of the USSR.

By V.E. Flint *et al.*

Translated by Natalia Bourso-Leland. *Princeton University Press: 1984. Pp. 353. \$65, £54.20.*

The Birds of China.

By Rodolphe Meyer de Schauensee.

Smithsonian Institution Press/Oxford University Press: 1984. Pp. 602. Hbk \$45, £35; pbk \$29.95.

A Pictorial Guide to the Birds of the Indian Subcontinent.

By Salim Ali and S. Dillon Ripley.

Oxford University Press: 1984. Pp. 177. £22.50.

Field Guide to the Birds of North America.

Edited by Shirley Scott *et al.*

National Geographic Society/David and Charles: 1984. Pp. 464. Pbk \$13.98, £8.95.

It is rare that readers of ornithological reviews are vouchsafed gratuitous political comment, but the production in one year of English-language field guides for the super-powers and the largest of the non-aligned nations can hardly pass unremarked. The problem is to know what this signifies — capitalist exploitation of new commercial opportunities or a shared concern for the natural world? Whatever the explanation, we can value each book for its own sake because all four of them make a worthwhile contribution to accessible knowledge.

A Field Guide to the Birds of the USSR was originally published in Russian in 1968 but soon dropped out of print and became, for practical purposes, unavailable in the West. This English-language edition has been updated by the senior author, Dr Vladimir Flint, in collaboration with advisers from the Massachusetts Audubon Society. A new introduction provides the Western reader with a very generalized account of the major habitat divisions of the USSR and their characteristic bird communities. The 750 individual species-descriptions comprise a summary of field marks, drawing attention to features aiding the separation of similar species, plus information on habitats, behaviour, nesting and distribution, with maps. The colour plates are good, and publisher and printer deserve credit for the standard of colour correction, which, when badly done, often mars good origination.

Though smaller than the USSR, China contains nearly 1,200 bird species, straddling as it does the palaearctic and oriental faunal regions. *The Birds of China* provides field descriptions of all adults, but lacks the information on habits so helpful to field identification. Distribution is

described in terms of provinces, which are mapped on the endpapers. The plates cover less than half the species occurring, and are based on museum specimens which are frankly described as being "of varying ages". Not unwisely, the illustrators — John Henry Dick, John Gwynne and H. Wayne Trimm — have largely adopted a somewhat cartoonish style, with little attempt to show precise plumage detail or jizz. The shortcomings of the work largely reflect the lack of information, and when one realizes that the artists and the author are based in the United States one must applaud the achievement rather than criticize. Sadly, there is as yet no edition published in China.

A Pictorial Guide to the Birds of the Indian Subcontinent shares many species with the previous two books, and with them serves to create a mutually supportive trilogy of guides. It is a useful work in its own right, however, the strongest point being the illustrations by John Henry Dick which cover all species, including females and some juveniles, plus flight illustrations of raptors, some waterfowl and seabirds. In comparison the text is disappointing, comprising little more than the briefest statement of habitat preference and distribution, derived from and cross-referenced to Ali and Ripley's ten-volume *Handbook of the Birds of India and Pakistan* published between 1969 and 1974.

All three of the books discussed above owe their present incarnations to American initiative or co-operation, and the pre-eminence of the United States in the compilation of ornithological reference works is underlined by the *Field Guide to Birds of North America*. This book is the fruit of work by four consultants, nearly forty editorial and production staff and thirteen main illustrators, but what sounds like a recipe for unmitigated disaster has produced a guide of remarkable scope and high standards and utility. The colour illustrations cover most plumage variations, enabling one to compare, say, juvenile crowned and yellow-crowned night herons or flying juveniles of the light and dark phases of skuas — sorry, jaegers! With, on average, four species per plate, there is adequate room on the facing page for helpful descriptive text and distribution maps of varying scale, enabling more precise definition of more localized species. In practical terms, the *Guide* has the virtue of covering the whole of North America in a single book, unlike the otherwise excellent Peterson volumes. It should also find a market on the eastern side of the Atlantic, for its usefulness in relation to those species we normally share as well as for the vagrants which regularly test European ornithologists each autumn. □

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Fish: relations and compositions

D. H. Cushing

Fishes of the World, 2nd Edn.

By Joseph S. Nelson.

Wiley: 1984. Pp.523. £52, \$44.95.

Marine Fishes.

Edited by V.P. Bykov.

Balkema: 1984. Pp.322. Dfl. 50, £11.50.

PROFESSOR Nelson's authoritative text, the updated edition of a book first published in 1976, describes the classification of fishes down to families and it lists the genera. There is an illuminating discussion on the nature of systematics, cladistic or synthetic, but no hint of the recent flurries in this area. Each order is defined in anatomical terms, as is each sub-order and family; in each description there are indications of ecological status. The drawings are very clear and simple, and are linked quite exactly with the anatomical description, while on the end-papers there is a helpful diagrammatic presentation of the hierarchy of the higher categories of fishes. Links to the fossil groups are well established and, indeed, the fossil genera are listed in the description of each order. There are 45 maps showing the distribution of a class or of families throughout the world.

The volume edited by Dr Bykov is superficially similar to Professor Nelson's, in that the fish species described from Russian catches are grouped by families within two super-orders, Selachioidei and Teleostei. There the similarity ends for this classification is a convenience, not an end in

itself, and the real point of the book is to list the chemical composition by species, region and season and by parts of the body. In certain cases methods of processing are given in some detail. There is no attempt to describe each species for the illustrative drawing is probably considered sufficient; such drawings are well executed and can be matched with those of Professor Nelson. So far as I can see the species are correctly named, and while the book is of somewhat specialist interest there are useful snippets of information scattered throughout the text.

As works of reference the two volumes serve different readerships. That edited by Dr Bykov will be used on far-ranging factory ships and in processing plants where an indication of processing quality of each species caught would be of considerable value. (The book, incidentally, also indicates how far the Russian fleet works away from its home waters, the Barents Sea, the Baltic and the north-west Pacific; in the Barents Sea, species and their chemical composition would be well known). There are two readerships for Professor Nelson's text. First, museum systematists will be glad to have the classification set out so clearly for those groups in which they are not experts. Secondly, fishery biologists whose trade is population dynamics should profit from the arrangement of orders; the volume should be in every library where fisheries are studied. For example I have used it recently to track certain genera unfamiliar to me in a study of the marine resources of the western Indian Ocean. □

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20-1-87
A. Galt

Building an umbrella for 1985

Next month's meeting between the United States and the Soviet Union is welcome. But the meeting will best succeed if its goals are modest, a standing commission on strategic arms in the first place.

NEXT year will get off to an interesting start. On 7 January, Mr George Shultz, the US Secretary of State, and Mr Andrei Gromyko, the Soviet Foreign Minister, are due to meet in Geneva to take up again the issues of arms control whose discussion was abruptly halted just over a year ago, when the Soviet Union walked out of the Geneva talks on intermediate-range missiles (INF) and strategic missiles (START). What is the chance that next month's meeting will make progress when so much has so recently gone wrong?

Three separate sets of auguries suggest that, this time, the two superpowers will have an incentive to agree. First, military costs are hurting both the superpowers; in the United States, President Reagan's hope of keeping the federal deficit within reasonable bounds seems yet again destined to be frustrated by Mr Caspar Weinberger's demands, while the Soviet government has signalled the high cost of defence by increasing the nominal proportion of its budget devoted to military affairs. Second, there has been a change of leadership in Moscow but also in Washington; Andropov has been succeeded by Chernenko and, just as significant, first-term Reagan, preoccupied with re-election, has been succeeded by second-term Reagan, likely (in the manner of his predecessors) to be anxious about what the history books will say of him. Then, third, both superpower governments have a common interest in reaching some tangible agreement on arms control before the third review conference of the Non-Proliferation Treaty fixed for next August; if they have nothing to show for their obligation to negotiate on strategic arms, they will earn another thorough drubbing from the non-nuclear signatories of the treaty, the continuation of which could easily be jeopardized. So something may emerge.

What? To begin with, not much. The announced objective of the Shultz-Gromyko talks is to find an umbrella under which serious negotiations may later take place. They are to be talks about talks, not substance. But that does not imply that nothing can be decided. What stands out from the experience of the past quarter of a century (since the Partial Test-Ban Treaty, signed in 1963 after five years of desultory negotiations) is that the pace of military development is too fast for the mechanisms of diplomacy. If the Soviet Union had not walked out of INF and START, at the end of 1983, in protest against the long-advertised deployment of intermediate-range missiles in Western Europe, President Reagan's star-wars speech in March this year might well have made them pack their bags.

Commission

So the obvious and urgent need, and the best kind of umbrella, is for a mechanism for the avoidance of surprises. Negotiations on particular measures of arms control are important, necessary and, indeed, long overdue. But there also needs to be a mechanism by which the superpowers can avoid startling each other, triggering off by either a walk-out from some continuing negotiation (at best), perhaps a more tangible hostile action. One of the benefits of ratifying the SALT II Treaty (signed in 1979) is that there would by now have been a standing commission representing both the superpowers to superintend the working of that agreement. The immediate objective, for Messrs Shultz and Gromyko, should be to establish just such a commission, but with wider terms of reference. There is no reason why an agreement to that effect

should not be realized in the first week of next year, or why a working commission should not be a going concern by the end of January.

But will there not be endless haggling about the terms of reference of such a body? There could, but need not, be. The practical needs are obvious. A standing commission (with an agreed timetable of frequent meetings) should be a forum in which one side or the other could disclose and explain strategic plans that would otherwise be surprising threats. Even the star-wars speech would have been less an irritant of Soviet susceptibility if the United States had been compelled to describe its plans in advance, explaining that, for the time being, star wars is a five-year programme of research, not even a development programme. A standing commission would also be a way of raising in private allegations that existing agreements or understandings are being violated. The United States has needlessly soured the international climate in the past year with public accusations that the Soviet Union has violated the unratified Threshold Test-Ban agreement (with another underground explosion marginally on the 150,000 tonne of TNT equivalent only a month ago), but has a legitimate question to ask about the phased-array radar being built in central Siberia, apparently in violation of the Anti-Ballistic Missile Treaty, signed in 1972, which allows such instruments only near a superpower's borders.

Modest goals

The chief practical value of a standing commission, however, would be as a means of agreeing in advance which specific strategic agreements are attainable. Part of the difficulty in the past decade is that the superpowers have locked themselves into over-ambitious negotiations. Although the breakdown of the Comprehensive Test-Ban Treaty in 1979 was at the time attributable to technical factors centring on verification, the real trouble was that neither US nor Soviet generals were ready for such a step. Nor are they now. Even the INF and START negotiations, which at various times in 1982 and 1983 looked hopeful, would have asked a lot of the generals and the US Senate, which would have had to ratify whatever agreements finally emerged. Far better, in these changed circumstances, that the two superpowers should informally decide privately on a sequence of modest goals that can then be quickly negotiated. It would be a tonic for everybody if there could be a succession of agreements on strategic (not always nuclear) weapons, however modest.

The ready objection to arms control by instalments along these lines is that its consequences will be undramatic. The non-nuclear signatories would for example spend next August/September complaining that nothing substantial had been achieved. Almost certainly, the Soviet Union will be looking for a quick dramatic agreement from beneath the umbrella now to be erected, if only to make plain that it has lost no face by walking out at Geneva a year ago, but also from a genuine if misguided belief that the United States is about to populate interplanetary space with novel and dangerous weapons. (That is at least five years and another presidential election away.) But a little reflection will show that even an attempt to return to INF and START would be imprudently ambitious but also intrinsically insufficient. Eighteen months of hard negotiation got nowhere and then, by

collapsing, did more harm than good; yet even these parallel negotiations were recipes for letting important issues (British and French nuclear weapons, the sense in Western Europe and the Soviet Union that intermediate-range weapons can have a strategic function) fall between the cracks. In retrospect, even unratified SALT II looks over-ambitious or, at least, too complicated to be workable.

So is there nothing to be done? Far from it. For starters, why not agree to ratify the existing treaties on the maximum size of underground explosions and that which outlaws peaceful nuclear explosions except with advance notice and inspection by the other side? So much could be agreed next month. So too could be the principles of a treaty to ban the testing of anti-satellite weapons. The United States, which has made one test with an air-launched rocket, says the Soviet Union has already deployed such a system, but its performance is known to be so poor as to be irrelevant. The next best bet would be an attempt to define some of the untidy edges of the aborted INF and START negotiations, the manner in which bomber aircraft should be traded off against missiles of long or shorter range, for example, or the relevance of battlefield weapons to the strategic balance.

Haggling of that kind, however necessary, will cut very little ice with the non-nuclear members of the Non-Proliferation Treaty, who will be looking for limitations, even reductions, of strategic nuclear weapons and, now, assurance that the region beyond the atmosphere will not become a battlefield. They will not have either by next August. But what they could be offered is a promise to work towards a much simpler agreement than either SALT II or the kinds of treaties likely to have emerged from INF and START. Why not simply settle for a limit on the number of nuclear weapons (separate warheads) that each side may retain, letting it decide for itself how they should be deployed? Verification is the obvious difficulty, which could be overcome by a necessarily private exchange of information on past production of fissile material. There might then follow an agreement to a cut-off on military production of fissile material, a scheme once dear to the French. And if the limit on nuclear warheads were small enough, the case for the deployment of whatever emerges from the star-wars research would melt away. Is all that too much to hope for? By recent experience, it is too much. The crucial question, which may be answered next month, is whether Mr Shultz and Mr Gromyko, and their political masters, will be able to change the tone of arms control. □

Agency two-step hazard

Trading the White House science office for a Department of Science would be wrong.

FEW things give a President of the United States such a feeling of accomplishment, with so little actual accomplishment, as a reorganization of the executive branch. Bureaux become departments, offices are merged, titles appear and disappear, but nothing changes. It is a generally harmless exercise, one that gives the illusion of increased efficiency while keeping the chief executive too busy to make a genuine nuisance of himself.

But there is something else going on with the proposals leaking out about President Reagan's plans for reorganization. Presidential science adviser George Keyworth is one of those suddenly enamoured of a cabinet-level Department of Science, apparently undeterred that this idea has been proposed more than a hundred times in the past twenty years, according to the Congressional Research Service. The proposed Department of Science would assemble under it all the research agencies of the federal government except agriculture, defence and those inextricably bound to a regulatory function, which means the Environmental Protection Agency and the Food and Drug Administration. So its components would include the National Science Foundation (now an independent agency), the National Bureau of Standards and the National Oceanic and Atmospheric Administration (which have always been somewhat out of place in the Department of Commerce), the National Institutes of Health, the

research components of the National Aeronautics and Space Administration (NASA) and of the Department of Energy (which department the Reaganites want anyway to eliminate) and the US Geological Survey.

Meanwhile, the murmurs are growing about plans drastically to reduce or even eliminate the Office of Science and Technology Policy (OSTP), the science adviser's office. The uncharitable (and probably wrong) interpretation is that Keyworth does not want any rivalry when he becomes the first Secretary of Science. A more likely interpretation is that even a vaguely independent office is a threat to the finely tuned political operations of the White House.

Although the idea of a Department of Science is probably less political (in the bad sense of the word) in origin, it is equally troublesome. And it is emphatically not in the category of cosmetic reorganizations. It has major consequences, not all of them good. To be sure, a cabinet-level department would, as they say in Washington, enhance the "visibility" of science; while OSTP, as an advisory body, can simply be ignored (and would be without the personal rapport that Keyworth apparently enjoys with Reagan), a Department of Science would have to be listened to in budget deliberations. And surely it would be better for science to have science budgets reviewed in the context of science budgets, not weighed against other pet projects that an agency may be pursuing. This would be especially true for space science, which must now compete within NASA against monstrosities such as the space station, created by NASA principally to perpetuate its institutional existence.

But the dangers are enormous. Unaesthetic though the present hodge-podge of science agencies may be, it serves a very real purpose in allowing expression for a diversity of views for the support of science. Putting them all together would render the entire federal research and development budget vulnerable to the sort of short-sighted political manipulations that, hitherto, have mercifully not afflicted more than one agency at a time. (Remember Research Applied to National Needs?) Worse, a cabinet-level department would be a perfect political plum for a political hack. Scientists may not always make good administrators, but they tend not to go far with half-baked schemes. The tradition of putting scientists in charge of science agencies has on the whole proved sound.

As for OSTP, it has obviously become a target for the political cosmeticians in the White House who ensure that the television cameras are always in the right place at the right time and that reporters are not given the opportunity to ask questions to which the President does not know the answers. As exceedingly careful as Keyworth has been to back the party line, even when that has meant making dubious statements about star wars and space stations, he is nonetheless regarded as an unnecessary risk by those who want all decisions made on the basis of political appearance. Similar reasoning explains the ambition to eliminate the Council of Economic Advisors, which has been on the hit list ever since Martin Feldstein dared to suggest that \$200,000 million deficits might have something to do with high interest rates. (The Council on Environmental Quality was all but eliminated four years ago, when the entire staff was fired and replaced by political familiars from Reagan's time as governor of California.)

OSTP, as now constituted, strikes a reasonable balance between window-dressing at one extreme and centralized control of federal research (which is what a Department of Science would do) at the other. The critics are right when they say it is weak because it is only advisory; that is what it should be.

Many who depend on one agency or another for their livelihoods will predictably oppose on narrow grounds any changes in the *status quo*. But they need not apologize for entertaining serious reservations about the proposal for a Department of Science, which should (and probably will) end up in the same place as it has on the 99 previous occasions it has surfaced. But the worrying aspect of these proposals is that they appear to be independent. OSTP could die an early death, and the plan for a Department of Science a lingering death in Congress. And then there would be nothing. □

Nuclear winter

US National Academy urges greater caution

Washington

A NATIONAL Academy of Sciences panel has concluded that* while the "nuclear winter" scenario — recently popularized by Carl Sagan — is a "clear possibility", the uncertainties are so great as to make it impossible to "put a number on the temperature changes or other climate effects". George Carrier of Harvard University, chairman of the panel, said he believed that both Sagan and Edward Teller, his most prominent critic, have been taking the results of recent calculations of the climatic effects of nuclear war "too literally", and that current atmospheric models can at best give "indications" of the magnitude of the global cooling that might follow an all-out nuclear exchange.

In testimony to Congress and in repeated public statements, Sagan has raised the



possibility that the fall in temperature following a nuclear war would be so great (37 degrees centigrade, according to his model calculations) and of such duration (several months or longer) that the survival of the human race would be threatened.

The panel's calculations produced similar results — a cooling of 20 to 25 degrees centigrade in the Northern Hemisphere, lasting for 6–20 weeks. But throughout their report, the panel stressed that uncertainties in both the assumptions and the models could vastly alter these numbers. The major uncertainty identified by the panel is the quantity of smoke that would be generated in fires set off by nuclear blasts. Small smoke particles are very efficient absorbers of solar radiation; the panel estimated that 180 million tonnes of smoke would be produced in a nuclear war that involved half the world's nuclear arsenal, and that more than 90 per cent of the incoming sunlight would be absorbed as a result. But the plausible range of smoke emissions is great, from 20 to 650 million tonnes; and because of the exponential relationship that determines total absorption, the light loss would diminish rapidly as the amount of smoke drops below 40 or 50 million tonnes.

Even with additional research, the panel

acknowledges, the climate effects of nuclear war will not be defined with great precision in the next few years. Indeed, because many factors depend on human decisions that can be changed at will, any calculation involves a significant measure of irreducible uncertainty.

The panel raised several apparently new questions about the nuclear winter phenomenon. One is the seasonal variations. The panel's global-circulation model calculations suggest that the effect would be much

Uncertainties of climatic change

THE academy report on nuclear winter studiously avoids the use of that term, even in quotation marks. The bulk of the report is concerned with the analysis of the assumptions on which calculations of the climatic effects of the nuclear war have been based. The committee has not constructed climatic models of its own, but has persuaded climatologists to re-run their models with different assumptions.

The committee's starting assumption is that 6,500 megatons of Soviet and US nuclear weapons would be detonated in northern mid-latitudes (compared with the 5,000 megatons in the Turco *et al.* baseline case). This hypothetical war involves warheads yielding 1.5 megatons or less, directed at hardened missile silos (one ground-burst each) and at economic targets (mostly in populated centres). But one of the variants considered includes an extra 100 20-megaton explosions.

For the study's baseline case, the quantity of the fine sub-micrometre dust carried into the stratosphere works out at 15 million tonnes (but could be twice as much), rather less than that calculated by Turco *et al.* But the addition of 20-megaton explosions makes it credible that 1,000 million tonnes of fine dust might be lofted there.

The academy study agrees with the conclusion of Turco *et al.* that soot carried into the stratosphere from fires ignited by nuclear explosions would have a more marked effect than dust on the flux of solar radiation absorbed in the atmosphere, chiefly because of the abundance of particles with sizes of the order of 0.1 micrometres in the smoke from burning fires.

The most important part of the report is the committee's careful discussion of the variables affecting smoke production, together with its review of the observations (from forest fires, for example) leading to the view that the size-distribution of the particles is log-normal. The conclusion is

greater in the summer than in the winter. And models of spring and summer conditions also suggest that a large transport of smoke particles across the Equator could occur in those seasons, spurred by solar heating of the debris cloud.

The panel's study was commissioned by the Department of Defense early in 1983, several months before Sagan publicized his findings. Although the report was completed almost a year ago, it went through an arduous review process before its release last week. A number of reviewers had strongly opposed the inclusion of any numerical results, no matter how strongly qualified, arguing that they would be misinterpreted. Indeed, several newspaper accounts reported the academy as having given its "seal of approval" to Sagan's conclusions.

Stephen Budiansky

that in the baseline case, 180 million tonnes of smoke would be produced (five-sixths of it from urban fires), that most of this would be distributed in the lower 9 kilometres of the troposphere and that very little would be injected into the stratosphere.

In the committee's baseline case, 250,000 square kilometres each of forest and urban landscape would be burned, with urban areas yielding 100 times as much smoke (3 g cm^{-2}) as forest. Uncertainty stems from possible variations not merely in smoke loading but also in the optical properties of the smoke particles and, in particular, the efficiency of the particles in absorbing visible radiation. The committee says that it cannot combine its estimates of uncertainty due to separate parameters because so little is known of the dispersion of the various sources of error.

Among the sources of error in the model calculations of the climatic effects of these smoke loadings, the committee singles out the impossibility, as things are, of incorporating into climatic models "the cloud microphysical processes that are primarily responsible for the removal of particulates from the atmosphere".

The results of the modelling studies carried out for the committee are qualitatively similar to those of Turco *et al.* Briefly, in the first few days after a nuclear exchange on the scale assumed, the Northern Hemisphere would contain several patches above which the optical depth of the smoke-laden atmosphere would exceed 20 (where the optical depth is the natural logarithm of the ratio of incident and transmitted intensities). Temperatures beneath the cloud of smoke would indeed be sharply decreased, in the worst case by a maximum of 35 degrees centigrade over six months but, with different assumptions about the speed with which smoke would be removed by rainfall, by a maximum of 15 degrees centigrade and to a substantial extent over a period of a month. □

Japanese universities

Private sector under new stress

Tokyo

JAPAN's private universities are making plans to get rid of their reputation for profligacy and mismanagement by voluntarily opening their account books to the public. Their poor image has recently cost them dear — three years of cuts in government subsidies have pushed more than half of them into debt and are forcing them to cut back on facilities and scholarships.

At present, Japanese law does not require private universities publicly to declare the source of their income or how it is spent, and much of their financial management has been veiled in secrecy. What has become visible has often been the result of a scandal: universities have faked reports of their expenses in order to receive larger subsidies, have taken huge "donations" to bend the admission rules for sub-standard students and have paid excessive salaries to directors and professors. Ugly internal feuds have also sometimes broke out: a short while ago, one board director was stabbed to death in a factional squabble over university management. Most of the scandals have been restricted to the smaller schools but even the leading universities have not been immune; one of the bigger medical schools is at present under fire for bending entry requirements.

Now, the Federation of Private University Associations, to which all but 4 per cent of the private universities belong, is preparing guidelines for the public voluntary announcement of financial reports. It is hoped that greater openness will give the universities the public support they need to halt the steady cuts in the government subsidies.

In the 1982 fiscal year, subsidies were frozen, in 1983 they were cut by 2.3 per cent and in 1984 by another 12 per cent. Less than 20 per cent of universities' expenses are now met by the government; a few years ago it was 30 per cent and the original plan was to finance 50 per cent of their expenses.

The result is that college deficits have hit new highs. The 82 members of the Japan League of Private Universities, including such important universities as Keio and Waseda in Tokyo and Dooshisha in Kyoto, have run up cumulative deficits that passed the 100,000 million yen (US\$416 million) mark at the end of the last fiscal year. With income not covering expenditure in 45 universities, cuts are being made in facilities and scholarships.

The government is already considering another cut next year, or at best a one per cent increase which will not even match inflation. There is little doubt that government officials, faced with Finance Ministry demands for further austerities, have taken advantage of criticisms of high salary levels in the private universities to single them out for cuts. But at the same time, their policy makes little sense in the light of the present

demographic situation and calls for increases in the number of researchers for Japan's "high-tech revolution".

As the children of the post-war baby boom reach university age, a massive increase in the number of university students is expected: the Ministry of Education is expecting an additional 86,000 enrolments a year in the early 1990s. But the boom will be short-lived — it will last only from 1986 to 1992, after which the population of 18-year-olds will fall dramatically. At present, 80 per cent of students go to private universities and the Ministry of Education wants to keep this constant; 80 per cent of the bulge is thus to be taken up by private universities. But far from preparing for the rush, private universities are now actually cutting back.

The only other way to pay for new facilities is to increase tuition fees, but there are signs that they are already close to what the market can bear. The average Tokyo family already spends 64,000 yen (\$267) a month on education. For those with a child

at a private university and another at a private high school, average expenditure on schooling comes close to 40 per cent of total living costs.

What seems likely in the near future is that private universities will simply take or more students without a substantial increase in staff or facilities. This will inevitably lead to a decline in standards. Private schools on average already spend per student only a quarter of what a state university can afford, the student-professor ratio is three times as high and classroom space per student one-third that in a state university. Few private universities can match state university standards.

These are problems that will clearly need urgent discussion in Prime Minister Yasuhiro Nakasone's Educational Reform Council, which has already begun its deliberations (see *Nature* 309, 739; 1984). Nakasone has himself stressed the need for increasing diversity in higher education, which he believes can be provided by the private sector. But the council has so far concerned itself largely with primary and secondary education and by the time it delivers its final report in 1986 the student boom will have begun. **Alun Anderson**

Gilbert resigns from Biogen

Washington

Dr Walter Gilbert, who gave up his faculty position at Harvard University to run the biotechnology company he founded, has now resigned as chairman of the company, Biogen. His action came after months of rumours about friction between Gilbert and members of the board, particularly those members with business experience.

Gilbert will continue to serve on Biogen's science advisory and supervisory



Dr Walter Gilbert with new chief executive Mark Skalesky

boards. A new chairman is expected to be chosen in February.

Biogen recently fired 16 per cent of its staff, a major cutback that some industry analysts interpreted at the time as a message to Gilbert of the board's dissatisfaction. Spokesmen for the company maintain, however, that the lay-offs were

part of a general "house-cleaning" designed to increase efficiency and check the company's overly rapid growth. Genex, another biotechnology company that has been growing very rapidly, announced a similar retrenchment this month.

Even with the lay-offs, Biogen will have grown 20 per cent this year, and further staff increases are expected in 1985. The company has a strong financial footing, with enough amassed capital to continue current programmes for several years before marketing its first products, according to Scott King, an analyst for Montgomery Securities. Although the company was able to raise only half of the \$60 million it had sought for a limited research and development partnership, it was able to put up the remaining \$30 million from internal funds.

Zsolt Harsanyi, vice-president of E. F. Hutton, says that some of the company's rapid growth may be attributable to the phenomenon, familiar on Wall Street, of "presentation of growth" — the desire of a company going public to give the appearance of rapid expansion.

The explanation may apply to Genex as well, although Genex seems to have run into more serious financial straits. A Genex spokesperson said that a number of costly design changes had been necessary in its phenylalanine plant, at present under construction in Paducah, Kentucky. Phenylalanine is the starting material for aspartame, the soft-drink sweetener. The lay-offs are expected to save Genex more than \$1 million a year. **Stephen Budiansky**

Proton decay

Italy's largest hole in ground

ITALIAN high-energy physics is now the proud possessor of the largest artificial subterranean cavity in the world. The objective is to mount a refined measurement of proton decay. But how this will be done will be decided only after an international meeting to be held in Rome next April.

The 100,000-m³ cavern, 4,000 metres under the Gran Sasso in the Apennines, is a by-product of the construction of what is claimed to be the largest pair of road tunnels in the world, linking Rome by autostrada to the Adriatic coast. The cavern has cost the Italian physics budget nothing, but has been built at a cost of 80,000 million lira (£32 million) by the Ministry of Public Works, partly as a means of providing jobs. But now it must be equipped, at a cost estimated at 80,000–100,000 lira.

The Gran Sasso experiment could become the most advanced proton decay measurement in the world, if only the money for instrumentation is forthcoming. According to one British physicist involved in another proton lifetime experiment, the Italian plans are "very futuristic" and the physicists may be "slightly embarrassed by the size of their hole". Proton decay is one of the testable predictions of "grand unified theories" linking all fundamental forces except gravity in a single theory; the existence of magnetic monopoles is one of many others.

Professor Antonino Zichichi, the ex-president of the Italian institute for nuclear physics (INFN), who planned the Gran Sasso hall, says that while some of the cost will be met from Italian sources, international support from Europe and the United States will also be necessary. Next April's meeting will debate the nature of the equipment to be installed in Gran Sasso, and specifically whether it should be 10,000 tons of water (to detect decay products such as pions, which travel faster than the speed of light in the medium, by their Cerenkov radiation), or some other form of particle detector.

Water is the cheapest solution, but another 10,000-ton water experiment has been under way in a salt-mine under Lake Michigan for a year without detecting any definite decays. The simplest grand unified theory is now generally considered ruled out by the Michigan result. So it may be necessary to equip Gran Sasso to detect, for example, slow kaons (too slow to make Cerenkov light in water) which result from proton decay in the more sophisticated grand unified theories now favoured.

Other proton decay experiments under way or planned in this area are:

- An Italian measurement 5,000 metres under Mont Blanc, which has been running since 1981 but with a small (150 ton) detector mass. After the Michigan result, it

now seems that the detectable lifetime of the proton will be beyond reach of this experiment, although it has recorded one unexplained event.

- An experiment in the Kolar gold mine in India, of similar scale and age, which claims three unexplained events.

- A Franco-German experiment in the Fréjus rail tunnel 4,900 metres under the Alps, between Turin and Lyon, using 1,000–1,500 tons of water crossed by thousands of detector-sandwiches. Although this experiment uses water, it may be able to detect kaons through decay products of the kaons themselves.

- A 1,000-ton calorimetric US-British

experiment that is under construction 1,800 metres deep in an iron mine in Minnesota.

Dr Wade Allison of the University of Oxford, who helped design the detector, says this Minnesota equipment will be well able to detect kaon decay modes, and that it may not be necessary to build detectors as big as 10,000 tons to get interesting results. All existing detectors are finding "inexplicable" events at the rate of 10 per year per 1,000 tons of detector, he says. These events are isolated in the chamber and do not, on the face of it, seem to be proton decays. But he suggests that they may be strange interactions of neutrinos emanating from cosmic-ray showers in the upper atmosphere or even experimental artefacts, but that the Minnesota experiment should decide.

Robert Walgate

High-energy physics

Italian budget to be doubled

THE Italian institute for nuclear research (INFN), which supports most of Italy's basic research in high- and low-energy nuclear physics, is likely to have its budget for 1985 more than doubled, to a total of 194,400 million lira (£85 million). This sum excludes the 50,000-million lira subscription to CERN, the international organization for nuclear research near Geneva.

This increase is a mark of the Italian government's will to build on Italy's important position in world high-energy physics, attested by Carlo Rubbia's recent Nobel Prize for the discovery of the W and Z particles. The increase will pay for Italian participation in HERA, the German electron-proton collider now under construction in Hamburg, a large proton lifetime experiment under the Apennines and other major projects including a study of a 50-TeV proton-antiproton collider (the ELOISATRON) which might be built in southern Italy.

This expansion in Italian high-energy physics makes a telling contrast with the falling support for nuclear and high-energy physics in countries such as Britain, where domestic spending has fallen by 55 per cent over the past decade. Moreover, the Italians have a lesson for the British; the problem lies with the physicists, who have failed to make their subject popular, and not with the politicians, says the now ex-president of INFN, Professor Antonino Zichichi. After "tremendous efforts" to popularize particle physics in print, in person, in theatres all around the country and on television, in flamboyant exercises which have sometimes brought him scorn among the less adventurous, Zichichi has fired the Italian imagination and won support for the first three years of his ambitious five-year plan for INFN, proposed more than a year ago. "You must convince the people first", says Zichichi, "and the politicians will follow".

This is why, Zichichi claims, the Italian parliament's budget committee and the lower house have already approved his plan. The senate is expected to follow soon. The figures give INFN 194,400 million lira next year, 220,000 million in 1986 and 230,000 million in 1987, even above the rate implied by the 1 million million total requested in the five-year plan.

INFN's present president, theorist Nicola Cabibbo, is cautiously awaiting the



senate's decision before making any statement, but Zichichi considers the senate vote a formality.

Apart from rousing popular support for high-energy physics, Zichichi also claims to have shown politicians the benefit of industrial involvement. Italian companies will be building many of the superconducting magnets and some of the electronics for the German HERA, thus putting Italy in a good position in a field where the technology is changing rapidly and industrial applications cannot be far off. The proton lifetime and other experiments in an artificial cavern under the mountain Gran Sasso has also provided employment in a region where jobs are scarce. During his campaign for a bigger budget, Zichichi has zealously exploited all these side-effects of high-energy physics. To those who have laughed at him, it seems he can now laugh back.

Robert Walgate

Genetic pesticides

Monsanto goes ahead with trials

Washington

THE Monsanto Company will shortly notify the Environmental Protection Agency of plans for the first field test of a genetically-engineered microbial pesticide. In doing so, Monsanto will become the first company to break with the convention whereby private companies have voluntarily sought approval for genetic engineering experiments from the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH).

The proposed test consists of planting corn seeds coated with a modified strain of *Pseudomonas fluorescens* expressing the gene for *Bacillus thuringiensis* toxin. This toxin has been used for many years as an insecticide, and protects growing corn plants from attack by black cut worm. *P. fluorescens* is found naturally in association with corn roots, and it is hoped that the modified bacterium could be a continuous *in situ* source of insecticide.

The company's decision to sidestep RAC was doubtless influenced by a preliminary injunction granted last spring by US District Court Judge John Sirica which prevents NIH from approving field trials of recombinant organisms without first preparing a formal environmental assessment on a field trial "program". The case is now before the US Court of Appeals. However, the injunction applies only to institutions that receive federal funds for research. Since Sirica's injunction was granted, the Environmental Protection Agency (EPA)

has moved to assert its jurisdiction in this area. The agency published an interim policy for field trials of modified organisms in October (see *Nature* 25 October, p.695).

Under the new EPA policy, 90 days' notice must be given for field tests of "genetically altered or manipulated" organisms. The test will be allowed to proceed if EPA raises no objection. Although EPA will not formally call on RAC to examine the experimental protocol, there is "total agreement" between EPA and RAC on the sort of data that are needed to support a notification, according to Dr Amy Rispin of EPA. EPA has representatives on some RAC subcommittees, thus ensuring "commonality of approach". Its actions in moving to regulate environmental releases of altered organisms have generally received high marks in the industry. EPA will require information on how the modified organism has been constructed, on the ecology of the host organism and on how release will be performed. Outside consultants will be employed to review areas where EPA has insufficient in-house expertise.

Monsanto has conducted preliminary greenhouse and growth-chamber experiments with *P. fluorescens* which indicate that it survives in corn plant roots for only 8-10 weeks if applied to the seed at planting. The modified strain does not even survive that long, according to Dr Robert Kaufman of Monsanto. Levels of expres-

sion of *B. thuringiensis* toxin are comparable with those in the source bacterium, and other metabolites also appear to be expressed at normal levels. *B. thuringiensis* toxin is specific to lepidopterous insects in its effects.

The test will be conducted at a research farm near Monsanto's headquarters at St Louis, Missouri, on a plot measuring less than one acre. The persistence of the modified bacterium will be monitored by tests specific to the toxin gene and the protein product. Kaufman points out that the bacterium is easily destroyed by household bleach, and that fumigation with methyl bromide could also be used to prevent the spread of the organism should it not behave in the test as expected. If the bacterium does decline after a few weeks, like the natural variety, it may be left in the soil over winter to see whether it will reappear spontaneously the following year. Kaufman will not be drawn on whether the new organism is likely to be economically competitive as a pesticide, saying the modified organism to be treated is still a "prototype".

Tim Beardsley

US food agency

Passing the hat?

Washington

THE NEW Commissioner of the Food and Drug Administration (FDA), Frank Young, seems to have taken to heart the spirit of the season — budget-cutting, that is — by circulating within the agency a proposal to defray some of FDA's research costs with private contributions.

The idea is apparently not a new one; each time it has come up before, worries over the obvious conflict of interest problem have resulted in a quick burial of the proposal. But given the enthusiasm of President Reagan and his appointees for "user fees" and for government-industry cooperation, the idea may survive at least a bit longer this time around. It has in fact received a strong endorsement from the scientific advisory board of the National Center for Toxicological Research, the FDA laboratory in Arkansas that carries out studies of the health effects of food additives, drugs and other substances.

As foreseen by Young, corporations and private foundations would contribute to a "blind trust" that would support the agency's research. Contributors would not be able to earmark their money for any specific projects. Presumably, the contributors from the food and drug industries would benefit in particular from research carried out by FDA on test methodologies. (In general, the manufacturers are required to perform their own safety testing on products they submit to FDA for licensing).

An FDA spokesman emphasized that Young had in no way committed himself to the idea and that, in any event, its implementation would require legislative approval.

Stephen Budiansky

Agencies vie for control

Washington

THE Environmental Protection Agency (EPA)'s competence to regulate environmental releases of genetically engineered microorganisms came under harsh scrutiny last week in Congress. At a hearing on biotechnology chaired by Representative John Dingell, EPA's assistant administrator for research and development, Bernard Goldstein, was grilled on an internal EPA memorandum asserting that the agency had "insufficient resources and no designated management plan for developing a cross-cutting biotechnology assessment programme in 1985".

The memorandum, by John Fowle, biotechnology coordinator in EPA's office of research and development, indicates that the office has 6.9 full-time equivalent staff and \$1.3 million dollars committed to biotechnology assessment in 1985. It concludes that 20 full-time staff and \$4 million would be required for a "credible" biotechnology programme, which would have 20-30 per cent of resources devoted to fundamental research.

Representative Gerry Sikorsky said that biotechnology assessment was low on

EPA's list of priorities and lamented that he "heard a lot of good talk" about regulation of biotechnology but that "there's little going on so far". Dr Goldstein said the biotechnology budget for fiscal year 1985 within the agency was more than \$1.6 million, but that the total budget for process regulation exceeded \$50 million.

EPA is not the only agency where attention is being given to regulation of biotechnology. Orville Wright, assistant secretary for science and development at the US Department of Agriculture, endorsed a proposed National Biological Impact Assessment Program to be located within the department. The programme would be research-based and would carry out "stepwise" assessments of proposed environmental releases of modified organisms. It is unclear how such a programme would be used by other regulatory agencies. Dr Bernadine Bulkley of the White House science office said the office would shortly propose a new interagency scientific review mechanism to ensure the best scientific advice is brought to regulators and to minimize overlapping jurisdictions.

Tim Beardsley

Laboratory animals

Research alarm at Israeli bill

Rehovot

ISRAELI researchers are concerned that a bill for the regulation of experiments on animals now before the Knesset will, if passed, seriously impede research.

Introduced by M.K. Eliahu Speiser, it prohibits experiments "except where they are essential". Exactly what will be considered essential is to be defined by Minister of Health Mordechai Gur after consultation with a special nine-member advisory committee, which would include two doctors, two veterinarians, a representative of the Association for the Prevention of Cruelty to Animals, the director of the Biblical Zoo, representatives of Tel Aviv University and Bar-Ilan University and, last but not least, a rabbi recommended by the Minister of Religious Affairs.

The bill calls on each research centre to appoint a person who will supervise experiments on animals and who would bear criminal responsibility — which could mean a fine of up to US\$17,000 and/or a year in gaol — if the regulations governing such experiments are violated by anyone at his institution.

Weizmann Institute Professor Benjamin Geiger, who heads the institute's committee of experimental animal users, agrees that experiments must be regulated but is not pleased with the bill as it now stands. What bothers him in particular is the make-up of the proposed advisory committee, which he believes should be composed primarily of active scientists with both a concern for the welfare of animals and an understanding of research needs. Such people would be aware of the pitfalls involved in any attempt to define "essential research". After all, Geiger notes, "probably not more than 10 per cent of all experiments yield applicable results, but if you don't carry out the whole 100 per cent you'll never reach the important 10 per cent".

Geiger also challenges those who would bar experiments on animals if the results are "only" of industrial or commercial significance. "Whatever importance one attaches to cosmetics, for example, it would be irresponsible to market new ones before they have been tested on animals", he declares.

Finally, Geiger does not think that the problem of animal mistreatment is likely to be widespread in Israel because major research centres here already abide by their own strict animal welfare regulations as well as those set down by the US National Institutes of Health and other grant-making agencies.

The head of the Weizmann Institute's "Helsinki committee" (for approving experiments involving human beings), Professor Irun Cohen, raises another question. "How is it possible", he asks, "to

have an outside body threaten scientists who use animals in their experiments with the imposition of a stiff fine and even a gaol sentence while allowing them to police themselves when it comes to studies on people?"

The Speiser bill is supported by Israel's Animal Liberation Group headed by Dr Andrew Menashe, a veterinarian with the Israel Society for the Prevention of Cruelty to Animals. In fact, Menashe thinks the bill does not go far enough, and he looks forward to the day when tissue culture tests and computer modelling will be substituted for all experiments on animals.

Like his counterparts elsewhere, Menashe also argues that tests on animals say very little about people. While noting that *in vitro* studies are adequate in some cases, in others, he argues, "you can't jump straight from Petri dishes to people".

Speiser agrees. Indeed, as an ex-biologist whose wife is a doctor, he realizes the importance of biological research, including that carried out on animals. "But", he declares, "these experiments must be properly supervised, and they aren't at present."

Nechemia Meyers

Academic open shop

Tokyo

JAPAN'S Ministry of Education is shortly to take steps to loosen up the academic system by enabling people from the non-academic world to become university professors.

At present, a strict set of ordinances, the university chartering criteria, makes it virtually impossible for anyone who has not followed a conventional academic career to become a professor or assistant professor in a state university. A professor must, for example, have either a doctorate or equivalent university research experience, or must have taught for more than a certain number of years at a university. Under the regulations now being planned, any person "recognized as competent in education and research" would be considered as eligible for a professorial post.

The change comes in response to the rapid growth in the number of highly talented researchers working outside the academic system — in research laboratories in private industry for example. Similarly, other trained people who could have something to offer the universities — judges, lawyers, ex-ambassadors and journalists, for example — will become eligible for university posts. Indeed, many such people already lecture in universities but are restricted to the status of "part-time instructor". If this injection of "new blood" proves successful, it is likely that further changes will be made to allow foreigners the same access to university posts.

Alun Anderson

New Year firs

Soviet *yolki* substitutes

CONSCIENTIOUS Soviet citizens should celebrate the coming new year without the traditional decorated fir-tree (*yolka*), the weekly *Literaturnaya Gazeta* urged at the beginning of October. A special correspondent from Armenia, Zorii Balayan, gave a glowing account of the 25-year campaign to outlaw the felling of fir-trees for *yolki* which resulted, last New Year, in not a single fir-tree being sacrificed to the festivities. The outcome, Balayan wrote, was an estimated saving to the economy of some 1.5 million trees which could now go to the paper and cellulose mills, the building industry or for the manufacture of vitamin C. Who will follow the Armenian example, asked the *Literaturnaya Gazeta*?

This is not the first time that *yolki* have been discouraged in what is now the Soviet Union. In 1914, Christmas trees were banned by the Holy Synod of the Orthodox Church as a "pro-German" influence (in spite of more than 200 years of cultural acclimatization since their formal introduction as part of Peter the Great's policy of "westernization"). They made a full come-back into Soviet life in 1941–42, when the necessities of war demanded the fostering of all national traditions, properly cleared of undesirable class and/or religious connotations.

Armenia, which is far distant from the North European traditions which engendered the mid-winter custom of decorating fir-trees, must have seemed a suitable area from which to eradicate the custom altogether. The first law forbidding the felling of *yolki* was passed in 1959, but in spite of numerous "educational" campaigns, felling of firs stopped only when every family could be provided with a reasonable plastic substitute.

Meanwhile, elsewhere in the Soviet Union, particularly in the northern and western republics, the annual felling of trees, including "poaching" from parks and nature reserves, still persists. A somewhat pathetic announcement broadcast on the Moscow home service by the Ministry of Light Industry of the Russian republic in mid-October offered conservation-minded citizens two variants of plastic trees, one with shaggy "snow"-covered branches and a new "fluffy" model, produced from granulated polythene by injection moulding. Both types had been awarded the state "quality mark", it was stressed.

How far the Soviet public has responded to this virtually unprecedented "commercial" will be seen only on New Year's Eve. It is noteworthy, however, that so far no educationalist has thought fit to revise the song Russian children sing around the tree, "All in the woods was *Yolka* born, All in the woods she grew!"

Vera Rich

European accelerators

Social scientists' attack on CERN

EUROPEAN high-energy physicists are so committed to the future of one laboratory, the European organization for nuclear research (CERN), near Geneva, that peer review is no longer a disinterested mechanism for judging the validity of CERN projects, two University of Sussex social scientists claim in a paper in the journal *Research Policy*, published today.

Rather, they claim, "systematic data" on past performance and future prospects of CERN, and laboratories like it, must be collected, and presented in a form "accessible not just to researchers in the speciality concerned, but also other scientists, policy-makers and even the general public". The authors, Ben Martin and John Irvine have, of course, done such a thing.

This paper* is the third and last of a series on CERN, and it concentrates on the decision-making that led to the adoption of LEP, the large electron-positron collider, as CERN's next project. But whereas the authors' previous papers have amounted to no more than historical research, the latest instalment strays into policy statements that are inevitably contentious.

The LEP decision was a mistake, Martin and Irvine imply, made possible by lack of objective external assessment in the peer-review system. LEP should be producing results by 1988, giving detailed data on the Z intermediate vector boson (and other phenomena such as the top quark). But a four-times cheaper machine, the Stanford Linear Collider (SLC), may produce similar data a year earlier, reducing LEP's usefulness, Irvine and Martin assert. And although the LEP decision was taken before SLC became a real possibility, it could have been reversed when SLC was approved, the authors have since argued.

Martin and Irvine's earlier studies show, they say, that a clear factor determining the scientific success of an accelerator is its uniqueness. Since LEP will not now be unique, it "raises the question... whether such major decisions, which have implications for other areas of basic science... should remain solely in the hands of the high-energy physics community".

The authors also say that the US studies of the 40-TeV "superconducting super-collider" (SSC), and similar but less-advanced studies of a "large hadron collider" (LHC) in the LEP tunnel, are a sign that another round in the intercontinental high-energy "arms race" is in the offing.

CERN director-general Herwig Schopper pointed out last week that discussions to avoid duplication of accelerators are already taking place in a high-energy physics panel established at the Versailles summit in 1982, and that SSC and LHC "are not projects yet — there are still various options". Moreover, Martin and Irvine's own work shows that one of

the main arguments being used for LEP in the late 1970s was its complementarity with US machines (in particular the Fermilab Tevatron, a proton collider in Chicago).

Even the late-starter (and earlier finisher), SLC, is complementary to LEP, Schopper argues, because it is primarily a machine physics experiment (to see if a

linear accelerator could be used effectively to produce colliding beam physics, a technique needed if even higher-energy electron colliders than LEP are ever required). Also, whereas SLC is confined to producing 50-GeV beams, LEP is designed to go on up to 100 GeV. "There will be some overlap in the initial phase, but that I think is a minor point", Schopper says.

Robert Walgate

*CERN: past performance and future prospects. III. CERN and the future of world high energy physics. *Research Policy* vol 13, no. 6, pp 311-342 (1984).

Campaign for research

Alliance for Science

THE Alliance for Science, a campaign for increased British spending on research and development, was launched in London last week. It has been mounted by three trades unions claiming to represent more than 100,000 scientists and technologists working in Britain. Stan Davison, deputy general secretary of the Association of Scientific, Technical and Managerial Staffs (ASTMS), said that the alliance had grown out of general concern over a considerable length of time. Government, education and industry were all to blame for the decline, he said, and workers in all these fields were represented by the campaign's constituent organizations, the Institution of Professional Civil Servants (IPCS), the Association of University Teachers (AUT) and ASTMS.

Mr Davison argued a case for innovative investment, one of the campaign's catchphrases. The organizers believe that this is an opportune time to launch the campaign,

with bodies such as the House of Lords Select Committee on Science and Technology and the Engineering Council asking that something should be done. The campaign will continue at least until the annual conferences of the political parties next autumn. The organizers are calling for a body for research to do the job that the National Economic Development Council does for the economy.

Ms Diana Warwick, general secretary of AUT, complained last week that the British government underestimates the amount of cooperation between industry and higher education, but said a "balance within universities between basic and applied research" is needed. She said that many developments in solid-state physics and X-ray crystallography originated from academic research programmes, and boasted that the biotechnology department of the University of Wales had turned a redundant dairy into an important centre for biotechnology.

The organizers are downcast that the number of graduate students fell by 20 per cent during 1979-83 because of restrictions on the science budget. And the annual growth rate in expenditure on research and development in Britain is now a third of that in Japan and a half of the comparable figure in West Germany. Twenty years ago, Britain led the field. "It is now very clear", Ms Warwick said, "from detailed reports and the University Grants Committee report, that the cuts have fallen on research rather than teaching. The equipment grant is over £16 million less than what it needs to be to keep pace with rising costs."

Bill Brett, assistant general secretary of IPCS, forecast at the alliance launching that there will be, between the British scientific civil service and the research councils, a further cut of 20 per cent by 1988. He attacked the "new phenomenon" in the scientific civil service of short-term contract research staff. The attendant insecurity was not conducive to satisfactory achievement. "Science has had a bad reputation", he said, "it has been associated with weapons and information technology of the worst kind." IPCS is eager to have people asking whether the United Kingdom is spending enough money on research.

Hugh Barnes

GLORIA scoops pool

A NEW model of the British side-scanning sonar GLORIA is to be built by the Institute of Oceanographic Sciences (IOS) under an agreement signed this week between the Natural Environment Research Council (NERC) and the US Geological Survey. Under the terms of the six-year agreement, NERC, which will lease back the sonar from the Americans, will survey the remaining five million square miles of the US exclusive economic zone, mainly in the Pacific and around Alaska. The mapping of seafloor features that may hold significant mineral deposits is part of a policy of assessing the long-term economic potential.

The total cost of the package, including charter of the research vessel *Farnella*, will be about £12 million. Although the Mark III GLORIA will be US-owned, the arrangement has the advantage of allowing IOS to use the existing version elsewhere. Completion of the new torpedo is planned for March 1986, with surveying commencing that summer. In the interim, mapping of the Gulf of Mexico will be carried out using the existing GLORIA.

Peter Gambles

Genetic engineering

Rifkin's foot in the door

Washington

AT a hearing this month, the US Court of Appeals gave some preliminary indications of how it will rule on the motion by the National Institutes of Health (NIH) and the University of California to lift a lower court ban on field trials of recombinant organisms. Although a decision from the three-judge panel is not expected for several months, the judges last week repeatedly interrupted attorneys for NIH, the university and Jeremy Rifkin — the antigenetic-engineering activist who obtained the lower-court injunction — with questions that suggested how they would vote.

The injunction was based on a finding by US District Court Judge John Sirica last spring that NIH must conduct a formal "environmental assessment" before approving the experiment proposed by Dr Steven Lindow of the University of California, in which genetically-altered bacteria were to be sprayed on potato plants to provide protection against frost. Even though the proposal was discussed at two open meetings of NIH's Recombinant DNA Advisory Committee (RAC) and published in the *Federal Register* for public comment, Sirica found that the law requires a formal document; he also concluded that RAC and NIH failed to consider possible environmental consequences of the experiment such as the effect of the bacteria on nearby plants and insects.

The preliminary injunction granted by Sirica also blocks NIH from approving any other field trials of recombinant organisms until NIH prepare an environmental impact statement on the full programme of field trials. NIH say that such a statement would take a year to prepare, and would be of little value because of the widely disparate nature of the field trials that are likely to be proposed. NIH have agreed, however, to prepare the much briefer environmental assessments on each field trial that is proposed. The University of California is continuing to challenge both the programmatic environmental impact statement requirement and the need for an environmental assessment on the Lindow experiment.

At the hearing, Judge George MacKinnon, who is generally considered to be the most conservative of the three, appeared to accept the government's argument that the RAC guidelines require the equivalent of an environmental assessment on each proposal submitted, whether or not there is a formal document with that name on it. And he several times sharply challenged Rifkin's attorney on the need for a programmatic environmental impact statement (EIS), pointing to precedents that established such a requirement only for programmes with a clearly cumulative effect.

Judge Abner Mikva, who is regarded as a liberal, suggested that if NIH were required to prepare such a statement, it would of necessity be vague and hypothetical; he told Rifkin's attorney that if his goal was closer scrutiny of the environmental consequences of field trials, "you might be better off without a programmatic EIS — frequently an agency will issue a broad EIS and then refer to that to cover all their sins".

If what appears likely happens — that the court allows RAC to proceed with approval on a case-by-case basis, but requiring an individual environmental assessment for each — Rifkin will still have gained the foot in the door that he is after. By establishing that RAC's review proce-

dures are subject to the National Environmental Policy Act — the law that requires environmental assessments and environmental impact statements — Rifkin will open the way for routine court challenges every time RAC approves a field trial; he will presumably argue that the environmental assessment in each case was not adequate or that a full environmental impact statement is required. Rifkin has said in several interviews that there should be a "moratorium" on field trials until a "framework" for assessing all the risks is established, a condition which he maintains is not possible with current knowledge. And he has also said that any environmental assessment should be done on a "worst-case" basis.

Meanwhile, RAC's staff is proceeding with preparation of an environmental assessment of the Lindow experiment.

Stephen Budiansky

European Parliament

Commission's budget rejected

THE massive vote by the European Parliament last week to reject the whole 1985 budget of the European Commission could mean trouble for the European fifth-generation computer project, Esprit, and for other scientific projects planned to grow or start afresh next year.

Esprit, for example, which links individual European computer and electronic companies in joint pre-competitive research projects, cost 45 million European accounting units (ECU) in 1984 (some £25 million). The programme was due to more than double to 100 million ECU in 1985. But under the rules governing the operation of the European Community without an official budget, spending must go on forward by "provisional twelfths" — each month the Commission will be able to spend no more than a twelfth of total spending in the previous year, in each individual line of the 1984 budget.

Of eight major programmes tabled for discussion at this week's meeting of research ministers (see *Nature* 15 November, p. 187), five are likely to receive no cash at all in 1985 until the battle with the European Parliament is settled. The non-nuclear energy programme, for example, may come to a total halt — its second programme finished 18 months ago, and since it had no official budget line in 1984, under the rules it cannot be funded in any fashion next year.

European parliamentarians, who have largely rejected the budget in a play for greater influence over the radical restructuring of the Community now under discussion among European heads of state, were last week attempting to play down the damage their decision has done to research. But the damage can be limited: some new programmes, it is pointed out in Brussels, must go through lengthy exercises of setting up expert committees and putting

out calls for tender. If the council of ministers meeting this week approves some of the programmes in principle, it may be possible to get through a large part of 1985 without spending an ECU, while nevertheless making some useful preparations to spend when the purses are filled again.

Robert Walgate

Hunting heads

THE Department of Education and Science is taking the un-British step of asking a head-hunting firm to suggest candidates for posts as the head of two research councils due to become vacant next year. The posts concerned are those as chairman of the Science and Engineering Research Council (which Professor John Kingman will leave at the end of August 1985) and chairman of the Natural Environment Research Council (which Mr Hugh Fish will vacate at the end of September 1985).

This unusual step appears to have been forced on the government by the dearth of acceptable names reaching it from the usual nominating bodies that constitute the old-boy network. The head-hunters, John Stork and Partners, have been retained only as advisers, and will be expected to provide Sir Keith Joseph, the Secretary of State for Education and Science, with a list of names from which to make a choice.

Part of the difficulty attending this is that the salary offered, that of an under-secretary in the civil service (now just over £30,000 p.a.) is too low to attract able people from industry and hardly enough to compensate senior academic scientists for the invidious responsibility of administering policies (or budgets) that must lose them friends. The usual compensation of a knighthood is not always a sufficient recompense, and cannot be traded in for heat and light. □

Symposium volumes

SIR — Anthony Watkinson's interesting and mildly cynical view of conference proceedings (15 November, p.201) represents one side of a very complex phenomenon. David Lodge, in his novel *Small World*, has explored some of the other activities of conference delegates. Judging from the locations of most US conferences and the attendant social programmes for both delegates and spouses, little time can be spent in the scientific sessions — hence the need for publication of the proceedings.

Whether or not proceedings should always be published, they are certainly wanted. The British Library Lending Division at Boston Spa has for many years tried to collect conference material and now holds about 200,000 volumes of proceedings. The library receives more than 250,000 requests a year for conference papers. A small percentage (less than 5 per cent) of these requests, but inevitably those causing the most trouble, are for papers allegedly presented at a conference but never published. Sometimes, however, the Lending Division holds papers which are never printed in the published proceedings.

I would urge that journal editors refuse to allow authors to quote references to conference papers that are not published unless this is made clear, and that organizers of conferences indicate in advance their publication policy. One way of tracing conferences, especially if the full title is not known for sure, is to consult the *Index of Conference Proceedings* published by the British Library Lending Division — a monthly keyword index cumulation, annually and quinquennially.

GARTH FRANKLAND

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SIR — Anthony Watkinson's article (15 November, p.201) on the publication of symposium volumes and conference proceedings explained why publishers like this kind of book. But he only outlined in general terms why many scientists are becoming increasingly hostile to such publications.

At a recent international meeting in the earth sciences, I was approached by no less than five publishers to produce a special issue or book of the symposium that I was convening. It became clear that the publishers were not particularly interested in the academic arguments for publishing this symposium (which happened to be of only average quality), but were largely concerned with printing paper and selling it at high prices to libraries.

The most important detrimental effect of the proliferation of symposium books may in fact be on journals. The best scientific journals are required both by commercial considerations and by duty to their readership to publish at regular

intervals. They therefore require a steady flow of high quality manuscripts reporting interesting and original scientific results. A healthy situation for the progress of science in general occurs when such journals have a sufficient supply of papers to be selective and to demand high standards, yet can publish a paper within a reasonable period of time. At present, the amount of high-quality research being carried out has reached a plateau and may even be declining in some fields. The symposium books, however, not only publish mediocre papers as implied in Watkinson's article, but also publish some first-class material. Such papers are removed from the reservoir of potential material available to the regular journals. In addition, they are not always reviewed very carefully — a process that is important even for good papers. These effects may well force some journals to lower their standards since they are usually obliged to publish a certain volume of material anyway.

There is no doubt that collections of papers on carefully selected topics can be outstanding contributions to the dissemination of knowledge. I believe, however, that scientists should be much more critical about agreeing to edit and organize symposium volumes. It is clear that most publishers will not agonize much about the various advantages and disadvantages as long as there is a profit to be made. Scientists are the only ones who will have a major effect by saying no more often.

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Science and miracles

SIR — In response to your leading article on miracles (*Nature* 310, 171; 1984), I think that there are at least two different views of science.

(1) Science as a parlour game, somewhat like chess. One sets the rules, decides who may play and who not, who is the most skilful at it, and even endows some with a certain authority. In such "science" one might state that there are no flying saucers and that miracles do not occur, by simply making it against the rules. Obviously such "science" is limited in its content and truth by the rules until someone changes the rules.

(2) Science as a quest for the truth or at least a working model of the truth. We do not set the rules but try to discover them. We try to achieve some confidence in what we find by analysis, experiments and observations, and not to discolour the truth with our prejudices.

It is reasonable to believe in miracles. The Creator of the Universe might well

create a miracle, and miracles have been reported through the ages up to the present times. (Consider the happenings at Lourdes, France.) Moreover, by the criterion of fewest serial steps and most parallel steps, the existence of the Creator is one of the most certain things in science. (Demonstration is beyond the scope of this letter, but consider the statement: "I think, which shows that God is".)

(Consider in this context the statement that this *cannot* happen because it violates So-and-so's law ...". One wonders: since when does So-and-so run the Universe?).

Much of the bickering in science might perhaps be ascribed to the following of the first view of science by people, rather than realizing that we are in the same boat of ignorance, that one cannot be absolutely certain of anything that one does not absolutely control, and that one is therefore dependent on what the Creator chooses to reveal directly or indirectly.

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UNESCO and VAT

SIR — The announcement by the British Foreign Secretary that he has served notice on UNESCO of withdrawal by the United Kingdom from the organization will come as no surprise to anyone, least of all to readers of your leading article (22 November, p. 293).

International considerations apart, the move has more parochial implications for the scientific community, as it comes at a time when strong rumours persist that the Chancellor of the Exchequer has proposed the imposition of Value Added Tax on printed matter. It is not perhaps widely remembered that the proposal was considered when VAT was first instituted but rejected because it infringes the UNESCO charter. Could Britain's projected pull-out from the organization weaken resolve at home to adhere to the principles of the organization? If books, and of course journals such as your own, were so taxed, the effects on education in its widest context would outweigh all the previously inflicted damage caused by previous cuts in financial support from the teaching of literacy up to graduate level. Beyond this, the restriction of information flow that would ensue as library committees stopped journal subscriptions would be paralleled by fewer personal subscriptions and the many learned societies that rely on income from this source would be severely hit.

Were this proposal to be formalized in a finance bill it would be too late to avert its implementation. The time for objections to be voiced is surely now.

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Technical analysis of IIASA energy scenarios

from Bill Keepin & Brian Wynne

Despite the appearance of analytical rigour, IIASA's widely acclaimed global energy projections are highly unstable and based on informal guesswork. This results from inadequate peer review and quality control, raising questions about political bias in scientific analysis.

DURING the energy crisis of the early 1970s, there was urgent interest in negotiating a transition from global dependence on fossil fuels to an ultimately sustainable energy system. To analyse such questions, a large-scale research programme was initiated at the International Institute for Applied Systems Analysis (IIASA) in Laxenburg (near Vienna), Austria. In 1981, the project issued strong conclusions and policy recommendations that were widely publicized in the professional literature¹⁻⁴ and the general press^{5,6}.

While working at IIASA, we have re-examined the quantitative analysis behind these conclusions. Much of the attention given to the published study appears to have been stimulated by the sheer magnitude of the project and by its claim to be a globally comprehensive and consistent study. The general conclusion was that world energy consumption would increase dramatically over the next fifty years. This would require major expansions of all energy supply options, including fossil fuels, and a major commitment to synthetic fuels and nuclear power. For example, nuclear power was required to expand at the average rate of one new power plant (1,000 MW) every four to six days for the next 50 years.

Our work has revealed various shortcomings both in the analysis itself and in its published presentation^{7,8}. Although the overall study involved more than quantitative analysis using computer models, this scientific aspect of the study has been given prominence as a claimed means of controlling bias in an inevitably ill-structured problem domain. The relationship between formal analysis (open to external review) and essentially inaccessible informal judgements in the construction and use of policy models has become an important problem, not only in the energy field⁹. Although informal judgement based on craft experience has been recognized as necessary, its existence poses a dilemma for quality control and normal peer review. Whatever the relative influences of formal and informal processes, analysis of the models themselves can help not only to improve technical standards, but also to expose the embedded informal commitments for external debate and evaluation. Greater elaboration in formal modelling does not mean reduced scope for informal subjective judgement — indeed the opposite may be true. This explication is

especially important in the IIASA energy study because the scenarios derived from the models continue to be used as definitive benchmarks in energy policy and associated analyses (for example of the global CO₂-greenhouse problem).

The major results of the IIASA Energy Program were based on comprehensive quantitative projections, or "scenarios", of the world's energy future for the succeeding 50 years. This article focuses on the analysis behind these scenarios, which were generated from a set of detailed computer models. It is found, in many cases, that the models essentially reproduce informally prescribed input projections that pass through the models unchanged. More importantly, despite claims of robustness, the scenarios are unstable with respect to minor variations in key input data. This was recognized in early sensitivity work that was discontinued and is not cited in later documentation.

These findings bring several conclusions drawn from the scenarios into question. They also raise more general issues relating to professional standards, peer review in non-experimental science, and the proper role of science in formulating public policy.

IIASA Energy Program

The IIASA Energy Program began in the wake of the 1973 oil embargo and lasted

seven years, involving more than 225 person-years of effort and a total research budget exceeding \$6.5 million⁷. More than 140 scientists from 20 countries participated in the study, and a broad network of collaborating institutions was established (including the UN Environmental Programme, International Atomic Energy Agency, Siberian Power Institute, and the US Electric Power Research Institute, National Center for Atmospheric Research and Stanford Research Institute). The final report was published in two volumes¹⁰ in 1981, entitled *Energy in a Finite World* (hereafter abbreviated as EIFW); an *Executive Summary* was more widely distributed¹¹. The IIASA programme is generally regarded as "the most ambitious such study carried out thus far"¹², and has had a considerable impact in scientific circles¹³⁻¹⁵ and among policy makers^{16,17}. The scenarios are routinely cited as prominent benchmark forecasts^{18,19}, and have served as a starting point for various subsequent investigations¹⁹⁻²¹.

The scenarios were central to the IIASA programme, and outweighed many other aspects of lasting value. Important results were produced from painstaking studies of global resource bases. Other valuable contributions include the logistic substitution model, and the IIASA work on CO₂, technological risk perception, and solar

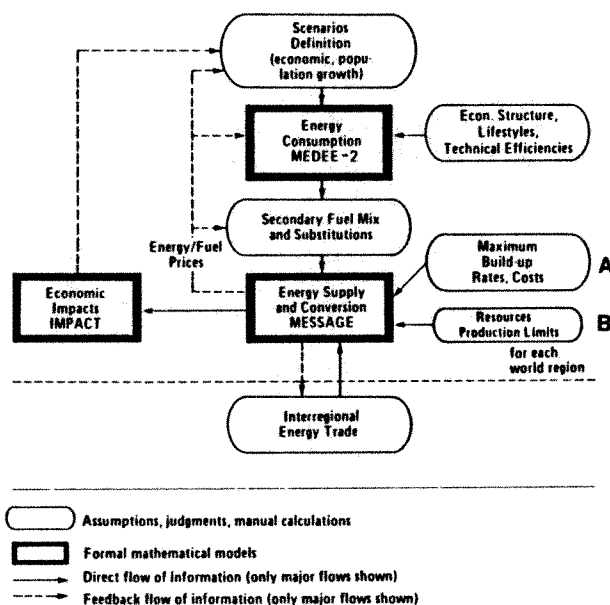


Fig. 1 The IIASA Energy Models (reproduced from ref. 10, p.401). Note distinction between formal models and "assumptions, judgements, manual calculations". Input data taken from ovals A and B are used to construct the "scenariettes" in Figs 2 and 3.

energy^{7,10}. In addition, IIASA provided an unusual forum for conferences and intercultural professional discourse. This article is therefore not a general critique of the entire IIASA Energy Program. Most participating scientists were not directly involved with the shortcomings reported here.

Our findings can only be summarized here (see refs 7 and 8 for details). This article focuses on methodology, and although there are policy implications, it neither endorses nor discourages any particular energy policy. Quotations are used extensively below to present the IIASA analysts' work in their own words. Unreferenced quotations refer to the *Science* article¹, and page numbers refer to EIFW, Vol. 2¹⁰.

IIASA energy scenarios

The stated purpose of the IIASA study was "to understand the factual basis of the energy problem, that is, to identify the facts and conditions for any energy policy". In this endeavour, "an explicit attempt was made to incorporate as many views and to be as objective as possible". To analyse the uncertain future, two scenarios ("high" and "low") were "constructed as a means of spanning the conceivable evolutions of global energy systems over the next 50 years" (p.656).

For this purpose, the world was divided into seven geopolitical regions¹, and individual scenarios were developed for each region. These were then aggregated to yield global scenarios, which were not intended to be forecasts, but rather realistic and pragmatic projections that "span a sufficiently wide range in order to incorporate the unavoidable uncertainties" (p.425). Conclusions were drawn from these to "provide decision- and policymakers with the information they need to make strategic choices" (p.800).

To ensure internal consistency in the globally comprehensive analysis, IIASA developed a set of mathematical models¹ which were the principal tool used in building the scenarios¹¹. These were of key importance in the analysis; according to EIFW, "the assumptions and results [from the energy supply model] represent, in some ways, the core of the energy studies reported in this book" (p.402).

A schematic diagram of the IIASA model set is reproduced in Fig.1. Note the distinction between formal models and informal procedures. Also observe that the major flows of information circulate in a clockwise fashion, linking the formal models together into an iterative loop. This is important because the models are extremely detailed, containing several thousand variables and parameters representing different facets of the energy system. These various quantities are reportedly controlled and adjusted by repeated iteration until a self-consistent scenario is obtained.

The iterative procedure begins with

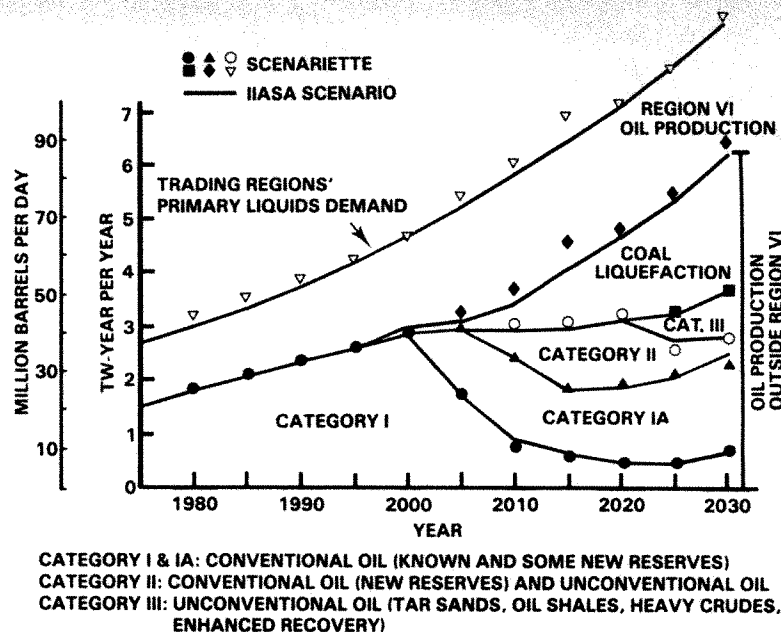


Fig. 2 World oil supply and demand projections (excluding centrally planned economies). Data points ("scenariette") are derived directly from inputs to IIASA energy models (Fig. 1). Curves (IIASA scenarios) are outputs from models. Close agreement reveals superfluous role of models. Compare Fig. 5 in ref 1.

initial assumptions about economic growth, which are combined with population projections and lifestyle parameters (in MEDEE-2) to produce projections of energy consumption from 1980 to 2030. These are fed into an optimization model (MESSAGE) which computes the least expensive supply strategy that meets the specified consumption levels, subject to constraints on resources and technology. This strategy is then fed into an economic model (IMPACT) that calculates requirements for capital investment, labour, land, water, materials, equipment and additional energy. Finally, "the main model loop is closed with IMPACT"²², whose outputs are fed back (via "the built-in feedback mechanism"²³) to modify the original economic growth assumptions. This completes one iteration cycle. Most feedbacks were manual, leaving room for an element of judgement, but the "flow of information is mechanized" (p.400), and the procedure is currently being streamlined²⁴.

In brief "the global high and low scenarios are the results of applying the model loop iteratively until satisfactory consistency was achieved"²², which in turn "required several iterations of the model set"¹¹. Note that the term "scenario", as used here, refers to the final quantitative results of a comprehensive mathematical analysis.

Role of the models

Despite sincere efforts to develop and implement the iterative procedure just described, it was never achieved. Major difficulties were encountered with the model IMPACT, which was finally "applied reluctantly and only at a place where no...

further modelling steps would be drawn from it"²⁴. Recently, it has been acknowledged that IMPACT served as a "monitoring model"^{7,8}, meaning that the iteration involved only the sub-loop between MEDEE-2 and MESSAGE, which had little effect. Indeed, only two examples of iteration are described in EIFW (pp.404-07), both involving minor adjustments in the heating sector (yielding 10% to 15% changes by 2030). Crucial variables and constraints (such as costs, build-up rates, resource extraction limits) did not enter the formal iterations. Moreover, closed-loop iteration of the three model loop was not performed, contrary to several statements in the documentation. Thus, macroeconomic constraints were not explicitly incorporated into the scenarios, and internal consistency was not quantitatively assured (a fourth model (MACRO) analysing macroeconomic effects (such as capital/output ratios) was said to be implemented earlier, but was later dropped⁸).

Given the lack of significant iteration in the model set, it is natural to ask what role the models played in calculating the scenarios. One approach to this question is to consider how the models compute scenarios from input data. For this purpose, direct comparisons of the inputs to models alongside their outputs may offer insight into the internal dynamic and analytic workings of the models themselves.

Such an approach has recently been carried out⁷. The procedure involved manual construction of a highly simplistic scenario, using only the model input data. This scenario is dubbed the "scenariette"

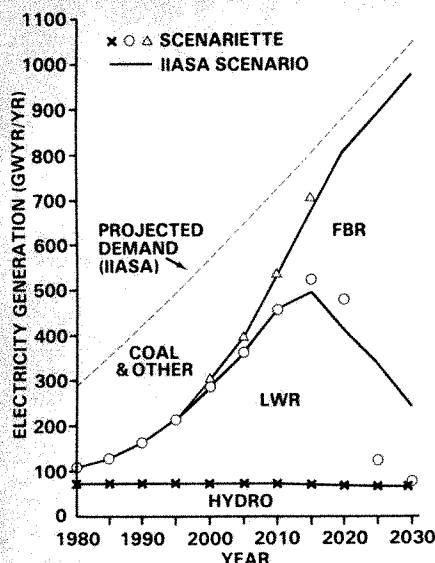


Fig. 3 Hydroelectric and nuclear power projections (for Western Europe, Japan, Australia and New Zealand). Points are derived from inputs to IIASA models (Fig. 1); curves are outputs. Rapid displacement of LWRs by FBRs is due to 2% cost difference (see Fig. 4).

to reflect the crudity of the analysis: usually it amounted simply to plotting a few sets of input data on the same graph. In some cases, it involved heuristic optimization and some minor accounting calculations (using a hand calculator), but no significant analysis (such as dynamic simulation, iteration and equation solving). Nevertheless, in many cases this back-of-the-envelope analysis reproduced the IIASA scenarios precisely, implying that the formal models are largely superfluous.

As an example, consider one of the most crucial aspects of the global energy system — the supply and demand for oil. A 50-year scenariette is constructed from time series input data taken from ovals A and B in Fig. 1. These data, which are external to the models and feedback loops, include prescribed constraint trajectories for rates of oil extraction and coal liquefaction in each region from 1980 to 2030. These are aggregated and plotted as points in Fig. 2. For direct comparison, the corresponding IIASA scenario is plotted on the same graph as a set of curves. Observe the striking agreement between scenario and scenariette. The differences between them are insignificant because precise numerical results "... must not be taken too literally. In fact, they must be seen as quantitative expressions of the more qualitative situations that underlie the scenarios".

Similar close agreements have been found in many other cases between the IIASA scenarios (both high and low) and the corresponding scenariettes⁷, including frequent identity (especially during the first 30 years). For example, Fig. 3 represents the results for hydroelectric power, light water reactors (LWRs) and fast breeder reactors

(FBRs) in a particular region (the models' only effect is to smooth the rapid phase-out of LWRs after 2015).

Although these findings raise questions about the models (discussed briefly below), the most important observation is that many of the principal scenario results are essentially prescribed by the input data — outside of the set of mathematical models. It is therefore natural to ask where these data come from, particularly since many of them are specific numerical projections extending to the year 2030. In EIFW (pp. 528–31), these data are described as "guess-timates", "rough average (sometimes consensus) estimates", "at best a zero-order approximation", or just simply "assumptions". Thus in most cases little empirical or theoretical evidence is supplied to substantiate the input assumptions, and few (if any) quantitative details are provided to indicate how the numbers were obtained. Thus, for coal extraction: "These constraints represent the roughly assessed composite and aggregate limitations of water, manpower, transport, environmental safeguarding, and so forth in each region" (p. 571). No details are given to show how these projected ceilings (on annual coal extraction) from 1980 to 2030 were obtained. For oil extraction, an outline is given (pp. 534–46).

A variant of Fig. 2 has been widely published^{1,4,10,11} as evidence for (1) the urgent need to develop synthetic liquid fuels (coal liquefaction), and (2) the necessity to exploit shale oil and tar sands, despite the tremendous environmental consequences that were not explicitly taken into account⁷. These conclusions are based primarily on the scenarios, which are now seen to be largely copies of input assumptions, which are in turn acknowledged to be informal guesswork. Thus, the analytical basis for these findings is inaccessible subjective judgement rather than formal calculation.

Sensitivity analysis

Modelling the global energy system is a formidable task, fraught with uncertainties that are greatly amplified when looking 50 years ahead. To complicate matters, long-term projections cannot be verified, and the time span of available data is too short to apply standard model validation tech-

niques^{25,26}. In this predicament, an indispensable tool is sensitivity analysis, which explores how projected results change in response to perturbations in input assumptions. This can help to identify major trends, key policy variables, and vulnerabilities, which is a principal motivation for modelling (rather than attempting to forecast). Moreover, careful sensitivity testing is essential when formulating robust conclusions from an analysis.

The importance of this was recognized for the IIASA scenarios, and it has been asserted that "the sensitivity analysis was done — at length"⁷. EIFW devotes a full chapter to alternative scenarios and sensitivity analyses, concluding that "Together, the scenarios, the alternative cases, and the sensitivity analyses should build a broad enough understanding... so that conclusions and recommendations for the energy transition [to a sustainable future] can be formulated" (p. 614). A number of recommendations and conclusions that are claimed to be robust have subsequently been broadly disseminated.

Since the 1973 oil crisis, it has been clear that future energy costs are extremely uncertain. Consequently, the IIASA analysis "did not start on the side of costs and prices". In fact, "the desire for data robustness dictated our decision to avoid an approach relying completely on prices" (p. 27).

"All of this leads to a belief (or hope) that the scenarios here are robust, that they can stand up against events whose impacts, in human terms, may be large" (p. 395). However, our analysis shows that the scenarios are highly sensitive to minor variations in assumed costs and other parameters. Summarized below are results from detailed sensitivity tests carried out for the low scenario (for the core model MESSAGE in particular⁷). Note that this scenario is now thought to be more likely²⁷.

Robustness of scenarios

Consider the assumed evolution of electricity generation costs (Fig. 4), most of which are held constant (in real terms) throughout the entire 50-year period. Although these fixed cost projections are highly unlikely, they would be acceptable for modelling purposes if the final results were insensitive to them. However, the exogenous projections shown in Fig. 4 determine much of the model behaviour.

For example, observe that the cost curve for LWRs undergoes a small step increase in the year 2005 due to an assumed increase in the cost of uranium. The model contains two categories of uranium: cheap and expensive. When uranium in the cheap category is exhausted, the model switches to the expensive one, raising the cost of LWRs by 7 per cent, as indicated by the "LWR step".

Note further that the FBR becomes available in the year 2000. Initially it is more expensive than the LWR, but then the LWR step occurs, leaving the FBR with a

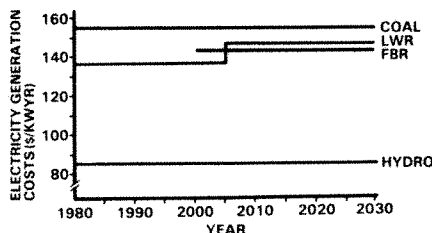


Fig. 4 Future costs of electricity generation assumed in IIASA scenarios. A small increase for LWRs in 2005 yields 2 per cent advantage for FBRs, producing major transitions to the breeder reactor (Figs 3 and 5a).

slight cost advantage (2.1 per cent). The result is that FBRs are built up as rapidly as possible, and LWRs are phased out (Fig. 3). This artificial feature of the model is the major factor determining the role of FBRs in all scenarios⁷. The electricity cost assumptions are the same in all cases, the only differences being in the temporal location of the LWR-step, which is governed by the quantity of cheap uranium available — a highly uncertain input parameter. An increase of a few per cent in the proportion of uranium allocated to the model's cheaper cost category delays the introduction of FBR by more than 30 years in some cases⁷. The IIASA scenarios were taken by many as strong evidence for the inevitable large-scale introduction of the breeder reactor¹⁵.

Other sensitivities are even more critical. Consider IIASA's 50-year projection for electricity generation in the United States and Canada (Fig. 5a). A 5 per cent increase in the assumed cost for the FBR eliminates its small economic advantage (Fig. 4), which causes the entire FBR contribution (Fig. 5a) to be replaced by additional LWR and coal capacity⁷. In another example, a 16 per cent increase in the assumed costs for nuclear power causes a shift to the radically altered projection shown in Fig. 5b (this case requires moderate increases in assumed coal extraction constraints after 2025⁷). Thus a mostly nuclear future is replaced by a mostly coal future (notwithstanding acid rain and CO₂) as a result of minor changes in input data whose values are known to be highly uncertain.

Slightly altered assumptions are thus seen to produce very different outcomes. This instability is intrinsic to the analytic structure of the supply scenarios, which means that robust future trends cannot be inferred from them. Nevertheless, several specific projections have been singled out and emphasized as robust conclusions^{27,28}. For example, "by 2030, nuclear power (LWRs and FBRs) has a total of 8.09 TWyr/yr for the high scenario and 5.17 TWyr/yr for the low scenario. Its relative share is close to 23 per cent in either case"²⁸. This requires that nuclear power stations be built at the average rate of one plant (1,000 MW) every 4 to 6 days for the next 50 years⁷. As seen above, this result is based on fixed relative cost assumptions that are not only highly fragile, but also are presumed to hold until 2030. Despite extreme sensitivity to tiny fluctuations in these assumptions (which have already occurred), this result is reported as a robust observation derived from the IIASA scenarios²⁸.

It is therefore of interest to review IIASA's sensitivity work. Three alternative scenarios were developed (nuclear moratorium, enhanced nuclear, and reduced demand), but these were not used as a basis for formulating robust conclusions and policy recommendations⁷. Rather, the high and low scenarios were used for this purpose, so it is particularly important to explore sensitivity in these cases.

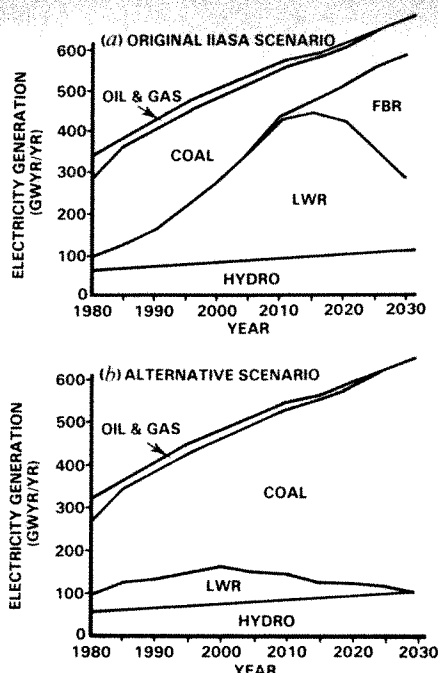


Fig. 5 Sensitivity to IIASA cost assumptions (Fig. 4) in United States and Canada, demonstrating structural instability in IIASA scenarios (which invalidates "robust conclusions" drawn from them). *a*, Original IIASA projection for electricity generation. *b*, Alternative projection, assuming 16 per cent increase in nuclear costs (and moderate increase in assumed coal extraction constraint after 2025).

After a general sensitivity discussion, EIFW treats "the more constructive consideration... of the possible variations among the relative price changes of new sources of energy" (p. 619). However, just three quantitative sensitivity tests are presented, none of which alters resource estimates or the critical relative cost ranking. The test for electricity generation assumes that all costs are doubled, thus shifting the curves in Fig. 4 upward, but preserving their relative positions. Hence the crucial sensitivities remain unexplored, and no further analyses are presented or cited⁷.

It might be supposed that the sensitivity problems we discovered are a realization of hindsight, but in fact they were recognized early in the IIASA Energy Program itself. Results similar to those shown in Fig. 5 were presented for a prototype model of MESSAGE in IIASA research memoranda published in 1974 and 1975²⁹⁻³¹. Despite the conclusion that "more work is needed in several directions"²⁹, these early findings are not cited or investigated further in the later reports and documentation⁷.

Technical discussion

Many of the technical shortcomings discussed above can be traced to characteristics of linear programming (LP) that are well known³². The single-objective LP model MESSAGE^{22,23} dominates the

IIASA analytical structure, largely because the full iterative loop was never effected. MESSAGE minimizes the total dollar cost of meeting a specified energy demand, subject to various exogenous constraints. The set of points that satisfy all the constraints is called the feasible region (or loosely, the set of all possible energy futures). A geometric representation of the LP concept is given in Fig. 6, illustrating the inherent stability problems. Given the large uncertainties in future costs of energy resources and technologies, cost minimization LP models are inappropriate for long-term robust supply projections^{7,32}. Efforts to overcome these difficulties are continuing (G.B. Dantzig, personal communication).

In many of the IIASA scenarios, the assumed constraints yield extremely small feasible regions — effectively single points. In these cases, the model behaves much like an identity transformation from the inputs (constraints) to the outputs (scenarios), as observed in Figs 2 and 3. The constraints are themselves arbitrary projections, subject to great uncertainty (to establish robustness would therefore require further sensitivity analysis — not provided in EIFW). Variations in the constraints can cause the feasible region to expand, thus magnifying intrinsic cost sensitivities (Fig. 6), to shift position or to disappear altogether, in which case the scenario ceases to exist. This last possibility may explain why the models' built-in environmental constraints, "although available, were not directly used" in the scenarios (p. 415). Since environmental constraints reduce the size of feasible regions²³, their inclusion could have eliminated the smaller feasible regions entirely, rendering the IIASA scenarios infeasible.

Certain caveats in EIFW hint at the provisional nature of the scenarios and conclusions, but these are not emphasized in articles and speeches. Recently, the minor bookkeeping role of the models in the subjective iteration process was acknowledged (Fig. 7). This figure was first published in 1983, two years after the publication of EIFW³³.

Conclusions

Two analytic results are reported here about the IIASA global energy scenarios. First, many crucial components of the scenarios are generated informally and supplied as inputs to the formal computer models, which then reproduce these projections with only minor alterations. Thus the models have not analytically "discovered" feasible energy futures. Indeed, despite the appearance of analytic sophistication and rigour, the models serve primarily as a static accounting framework for displaying the assumptions of the analysts.

Second, the supply scenarios are strongly dependent on assumed cost and resource estimates (for example, the projected expansion of the fast breeder reactor

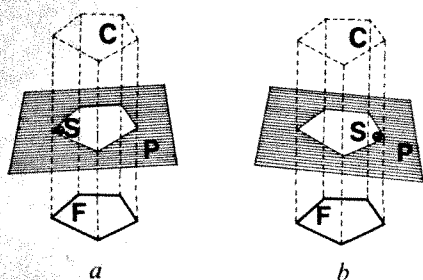


Fig. 6 Geometric representation of linear programming. *a*, Feasible region (F) is interior of polygon. Above F lies plane (P) representing total cost. Boundaries of F are projected vertically upwards (through P), defining constraint column (C). Small ball (S) is released on P inside C, and rolls to lowest point, defining optimal (least-cost) solution. *b*, Slight changes in cost assumptions tilt plane P in different direction, shifting solution from one extreme to another. This effect is observed in Fig. 5.

is due to a 2.1 per cent cost advantage introduced after 2005). Small changes in these highly uncertain estimates produce radically altered supply strategies. This inherently brittle structure precludes the possibility of drawing robust conclusions from the supply projections. Nevertheless, several conclusions have been drawn and widely publicized as robust findings, including massive expansions of fossil fuels and nuclear power.

In addition to these analytic results, various problems are found in the documentation. Early work revealing serious structural instability is not cited, and standard sensitivity tests are not included. The study's own publications have led outside scientists to believe that the formal models involved dynamic analysis and iteration for control of assumptions and genuine validation of the scenarios, rather than limited arithmetic checking. Only recently has the minor bookkeeping role of the models been clarified.

The IIASA energy study claimed to reveal a narrow window of feasible energy futures (for example, "Our scenarios are globally comprehensive and allow for no escape", p.785, original italics). Now, just three years later, a broad spectrum of possible energy futures is widely recognized^{25,34,35}. However, the IIASA conclusion did not result from an objective examination of possible future alternatives, but rather from embedded institutional and sociopolitical assumptions that pervaded the analysis^{8,9} (for example, expansion of capital-intensive supply technologies was emphasized, while options for reducing energy demand were largely ignored). Although the study claimed to focus only on factual aspects rather than social and political dimensions, this artificial division confined the resulting scenarios to a given institutional framework, thereby precluding full exploration of the range of possible futures. Scenarios

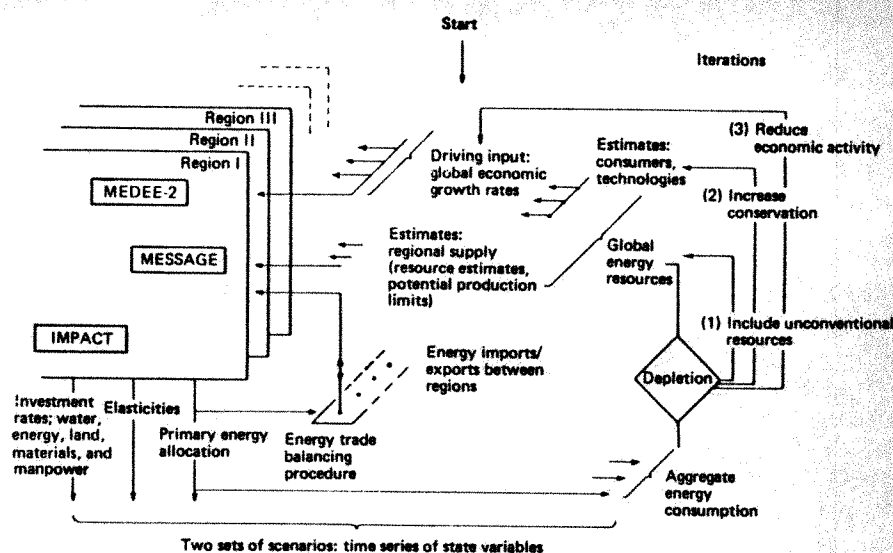


Fig. 7 New representation of scenario writing process³³, first published in 1983. Note minor role of models.

are rarely presented as forecasts, but they are frequently treated as such, even by their authors³⁶. The danger is that they will be misconstrued as inevitable, and used to justify policy decisions³⁴.

Much work of lasting value has been produced at IIASA, from both inside and outside the Energy Program. These important contributions have unfortunately been overshadowed by the energy scenarios and associated urgent policy recommendations. (For example, "what must be emphasized is the advent of... synfuels and the fast breeder reactor... the most precious resource is time".) Both the urgency and specific recommendations were evidently based on opinion rather than objective robust analysis, as claimed. One important lesson is that the most exquisite formal analytic modelling still embodies informal assumptions (often about sociopolitical values and insti-

tutional behaviour) that affect what technical outcomes are conceivable. Peer review of formal models can expose these assumptions for external debate and evaluation⁷. Indeed, rather than attempting to identify objective policy truths, perhaps a more realistic role for policy modelling is to explore origins and consequences of different social and institutional assumptions^{35,37,38}. Such an approach would embrace (rather than deny) the interpenetration of science and politics in policy analysis.

We thank IIASA for supporting this work, and the many individuals who helped us, especially Valerie Jones and Mike Thompson.

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Where now with nuclear winter?

The US academy's predictable plea for more research should be granted if people are that worried. But the real need is for steady nerves — and atmospheric physics.

THE US National Academy of Sciences' report *The effects on the atmosphere of a major nuclear exchange* (see p.683, this issue) turns out to be what it always had to be, a first or, rather (*pace* Turco *et al.*, *Science* **222**, 1283–1292; 1983), a second approximation to reality. The original account of what might happen if the atmosphere is loaded with soot from the fires ignited by 5,000 megatons of TNT in nuclear explosions is qualitatively confirmed, but hedged around with so many qualifications that a null outcome could well be compatible with the academy committee's analysis. Nuclear winter, in other words, would almost certainly be less severe, less widespread and shorter-lasting than Turco *et al.* originally declared and might even be indistinguishable from nuclear summer (which is not a pleasant prospect either). That, no doubt, is why the academy's committee has studiously refrained from using the term "nuclear winter", which is a pity; the phrase is too graphic to be ignored.

As things are, the new report will be mulled over by specialists in the field for its review of what is known of problems as different as the size-distribution of particles of smoke, the mechanisms by which the fireballs of nuclear explosions loft material to high altitudes and the ways in which climatic models may have to be refined to accommodate the needs of those who would model nuclear winter. (Cloud formation, as always, is a bugbear, what happens at the edges of a cloud a novel problem.) Latecomers in the field may, for the time being, be perplexed to know what more there can be to say, but no doubt reflection and ingenuity will enable them to think of something.

In passing, however, it must seem to many people odd that the nuclear winter subcommittee of the International Council of Scientific Unions (ICSU)'s Scientific Committee on Problems of the Environment (SCOPE), which is due to publish its own study next June, should have been able to afford time for a five-day workshop at Bellagio to draft and put out a statement which, on the face of things, will compromise the impartiality of its own work. The statement says that the prevention of nuclear war is a challenge for mankind (true), that there is "no hope" that technical innovations such as "weapons systems in space" will provide "clear superiority or significant protection" (which is probably true) and that

everybody must be "aroused" to the threat of nuclear war and to "the possibility that in some circumstances one of the results may be what has come to be called nuclear winter . . .".

The notorious ineffectualness of manifestoes apart, the danger of such statements is twofold. First, they give the impression that the issue has been decided before the study is complete. And, second, by giving needless offence to people who disagree, however wrong-headedly, with steps in their argument such as the assertion that space-based defence against ballistic missiles is impossible, they diminish their potential influence. That such a distinguished group of people (which includes Lord Zuckerman, Professor Charles Townes and Professor Abdus Salam) should be predicating its proposals for the avoidance of nuclear war on "fundamental changes in international relations, especially . . . between the Soviet Union and the United States", for all the world like old Keir Hardie socialists predicating social equality on "a change of heart", is nevertheless remarkable. The more serious problem of arms control is to avoid nuclear war even when the superpowers are daggers drawn.

This prompts one of the three general questions raised by the National Academy's deadpan text (which lets slip a flicker of emotive prose only in its last sentence, "one can ask whether even now the full range of physical consequences . . . of nuclear warfare is within our comprehension") and by the way in which the concept of nuclear winter has been used this past year. Politicians have embraced the idea (see *Nature* 13 December, p.593), usually with the best of motives. Technical people have followed suit, not for reasons that are political in the partisan sense but apparently in the belief that the threat of nuclear winter will persuade governments towards effective arms control. The risk is that such an incentive can be no more substantial than the threat. Some have further endangered their position by using the same bogey-man against extraneous targets as when Professor Paul Ehrlich told a BBC radio audience the other day that the British government's arrangements for civil defence were "nonsense", perhaps not knowing that those plans are justifiable only against a few bombs not an attack that could cause nuclear winter.

One of the technical community's admirable achievements, in what other com-

munities call the nuclear age, is its stiffness of purpose about the need somehow to contain the threat that a third nuclear weapon, then perhaps a fourth, and so on, might be used in anger. What those who work the nuclear-winter lobby overlook, or even reject outright, is that occupants of other lobbies, even the star-wars bunch, are equally steadfast in saying that their objective is to avoid nuclear hostilities. Logic-chopping in such circumstances serves little purpose (but in the 1950s it persuaded nuclear governments that the risk of genetic defects from fallout was too great to allow unrestrained nuclear testing in the atmosphere). The sad truth is that trying to bridge this gap is a plainly more formidable task than that of winning more general huzzas by identifying a hazard that others have not seen.

In the circumstances that have now developed, it is hard to know what should be done. The National Academy report asks that there should be more research. It does not say how much more. What the report has done is to identify a series of important and interesting problems in atmospheric physics that, in their own right, deserve attention. Dealing with them under the contrived rubric of nuclear winter rather than atmospheric physics will be artificial and even uneconomic. The price worth paying will depend on how deeply and generally ingrained is the belief in the certainty of nuclear winter after nuclear war. The ideal would be to spend the extra on atmospheric physics.

What should be done? Bigger and better models are not the immediate need. What the argument about nuclear winter has vividly demonstrated is that future models need better ways of dealing with physical processes in the atmosphere. After a century of research (since C.T.R. Wilson), the dynamics of cloud formation is only poorly understood, yet clouds provide the rain that cleanses the atmosphere of nuclear smoke as well as other things. Taking account of discrete clouds (as distinct from average cloudiness) in climatic models remains a puzzle. Dealing with what happens when contiguous columns in the atmosphere differ sharply in constitution (as at the edge of a cloud of nuclear smoke) is an interesting challenge. It may not be one of those "fundamental and moral challenges to all of humanity" referred to in the preamble to the SCOPE subcommittee's manifesto, but it might help people to sleep more easily in their beds. **John Maddox**

Astronomy

Draughtsmen of the constellations

from David W. Hughes

TO THE astronomically-initiated stargazer, six hundred or so of the brightest stars are dragooned into an array of constellations. For anyone who has wondered why, when and by whom this cartographic system was drafted, Archie Roy of Glasgow University provides some answers in *Vistas in Astronomy* 27, 171; 1984.

First, bear in mind that an observer living at latitude 0°N not only never sees the south celestial pole but also finds that stars with declinations between -90° and -0° never rise above the horizon. Moreover, the celestial poles are not fixed but precess about a circle of radius 23.5° every 26,000 years. It is, then, not difficult to establish the era and the domicile of the original draughtsmen. There is a patch of sky in the southern celestial hemisphere which has no ancient constellations, even though it is spattered with bright stars, and whose angular radius of avoidance is about 36° . This means that the originators of the constellations must have lived somewhere on the longitude line 36°N . And, allowing for precession, the centre of the zone corresponds to the south celestial pole of around 2,500 BC.

Why was the constellation system devised? The clues come from the work of Hipparchus (125 BC), Eudoxus (409–356 BC) and Aratus (315–250 BC). Aratus was a poet who, at the instigation of King Gonatus of Macedonia, wrote the *Phaenomena*, a poem embodying the work of Eudoxus. According to M.W. Ovenden (*Philosophical Journal* 3, 1; 1966) the daily and seasonal behaviour of the constellations discussed in this work are commensurate with a date of $2,600 \pm 800$ BC and a latitude of $36 \pm 1.5^\circ\text{N}$. But Roy's re-analysis of the poem, concentrating on its description of the positions of the celestial equator and the celestial tropics of Cancer and Capricorn, leads him to date the description to $2,000 \pm 200$ BC; Eudoxus seems to have been writing not about the sky that he could see but about the sky as it was 1,600 years earlier. Neither Eudoxus nor Aratus bothered to check that their poetic picture agreed with the Greek sky of 400–250 BC: they simply reproduced previous work. Hipparchus did check, and the discrepancies probably led him to discover polar precession.

Eudoxus was writing about people who had a sophisticated astronomical outlook which encompassed an Earth poised in space with the heavens revolving about it on a bipolar axis, although one pole was not visible. The constellation system seems to have been designed primarily as a navigational aid for sailors. Non-circumpolar constellations (those with declinations between $+0^\circ$ and -0°) always rise and set

at a specific compass point on the eastern and western horizon. A ring of constellations with the same angular distance from the celestial equator can be used to indicate a specific compass setting throughout the night and would have been a great navigational aid at a time when there was no acceptable pole star.

So who were the original draughtsmen? Four groups have been suspected –

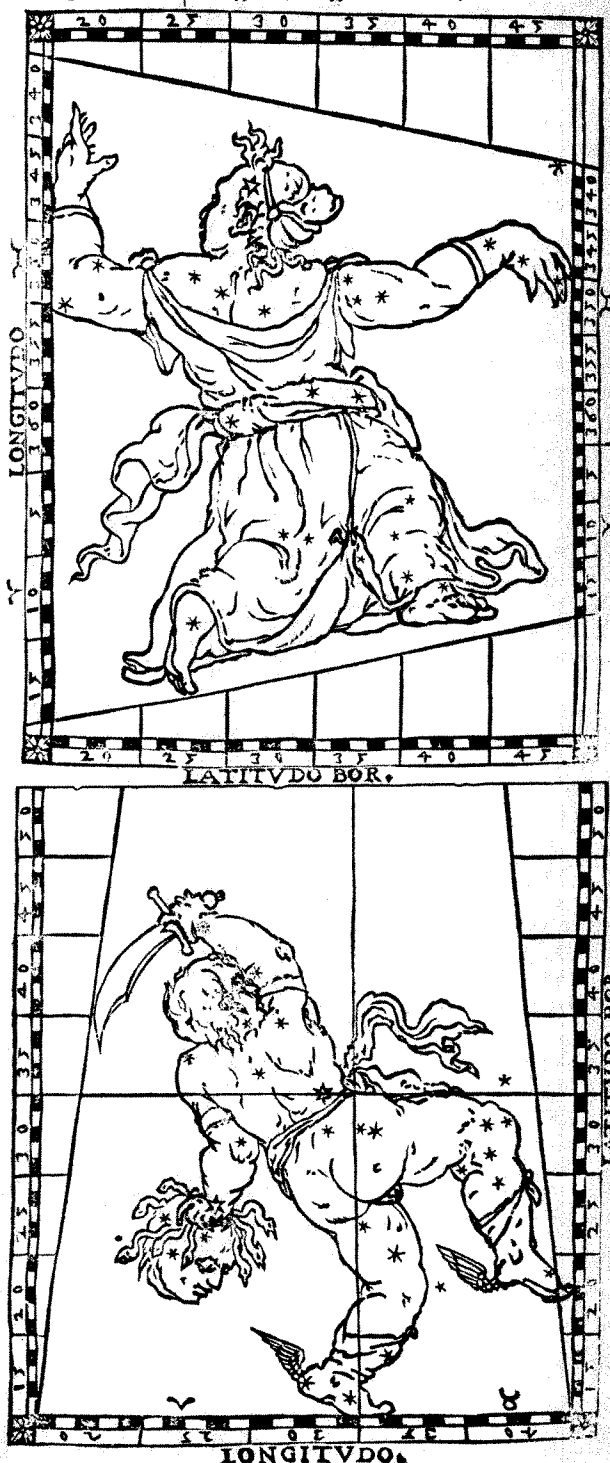
the Phoenicians, the Egyptians, the Babylonians and the Minoans. The Phoenicians can be ruled out because they prospered too late, from about 1500–500 BC. The Egyptians, despite their sophistication and their role as tutors to early Greek science, simply lived too far south. The Babylonians are stronger contenders: the Sumero-Akkadian people of the Euphrates region were great believers in astrology, and the movement of planets through the Zodiac was used for calendrical and religious purposes. The records left on their clay tablets leave us in no doubt that as far back as 2,100 BC they were using a system of constellations essentially similar to that given in Aratus's poem. Moreover, navigation was important to the Babylonians for their links with India and South Arabia, centred on Bahrein (ancient Dilmun). On the other hand, their sea traffic, was closer to the equator than the 'observer's latitude' derived from Eudoxus.

This leaves the Minoans, who lived on Crete and the Cyclades, and traded from there with Egypt, Greece, Cyprus,

Syria and Mesopotamia throughout the third millennia BC. Roy considers that it was the Minoans who borrowed and developed early Babylonian ideas and transformed them into a complete celestial sphere of constellations for navigational purposes.

But why was the Eudoxian sky 1,600 years out of date? A simple solution is proposed. With the complete destruction of the Minoan civilization around 1,450 BC by the volcanic explosion of Thera (Santorini), their astronomical ideas and constellations were frozen in time. □

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Andromeda (top) and Perseus by Giovanni Paolo Gallucci (1588). From George S. Snyder *Maps of the Heavens* (Andre Deutsch 1984), to be reviewed in *Nature*.

Atmospheric physics

Interaction between whistlers and radiation belt electrons

from M.J. Rycroft

ANYONE using a radio receiver during a thunderstorm is familiar with the fact that lightning produces radio waves. What is not so well known is that some of these radio waves, those at audio frequencies of a few kHz, get trapped in ducts aligned along the Earth's magnetic field and bounce back and forth ('hop') along the dipolar field lines. The dispersive nature of the medium through which the waves travel ensures that, in general, the higher frequency components travel faster than the lower. When picked up on a broad band receiver, the waves are heard as a succession of descending whistles (hence 'whistlers'), the rate of descent decreasing on successive hops. On page 740 of this issue Voss *et al.* report the first satellite observations of another direct consequence of lightning flashes: the dumping of energetic electrons from the magnetosphere into the upper atmosphere¹. Such phenomena have geophysical and astrophysical relevance.

The van Allen radiation belts contain electrons and ions that are trapped by the Earth's magnetic field, accelerated to energies of some tens of keV by a variety of plasma physical processes which occur in the magnetosphere, preferentially on the night-side of the Earth, and associated with auroral substorms. Theoretical considerations of the loss of electrons from the belts have focussed on the cyclotron resonant interaction between whistlers, the frequencies of which are below both the plasma frequency and the cyclotron frequency, and energetic electrons travelling in the opposite direction along a geomagnetic flux tube. The condition for cyclotron resonance is that the electron 'feels' the wave Doppler-shifted up to its cyclotron frequency. In the equatorial plane, the phase velocity — and also the group velocity — of whistler mode waves propagating along a geomagnetic flux tube in a duct of slightly enhanced plasma density is a minimum. Thus, the equatorial plane is where cyclotron resonance occurs with electrons of the least energy.

The first observation of such a wave-particle interaction in the magnetosphere was serendipitous; during the flight of a Petrel rocket, carrying Geiger counters and launched from the Outer Hebrides, a transient increase in the intensity of energetic electrons having pitch angles between 60° and 120° was recorded (Rycroft, M.J. *Planet. Space Sci.* 21,239; 1973). The increase, by a factor of 20 above the quasi-steady intensity observed throughout the remainder of the flight, occurred in 0.8 seconds and was simultaneous for both >45 keV and >110 keV electrons. About

half a second later a two-hop whistler was recorded on the ground. During the period of enhanced electron intensity, the entire duration of which was only about six seconds, four-, six- and eight-hop whistlers were also received. From an analysis of their spectrogram, it was concluded that the whistlers were ducted through the magnetosphere along the $L=3.3 \pm 0.1$ field line. The electron density in the equatorial plane, N , was found to be $330 \pm 10 \text{ cm}^{-3}$, a value that is characteristic of conditions within the plasmapause.

It was suggested that the association of whistler and electron intensity was not coincidental but the result of a cyclotron resonant interaction between energetic electrons and whistler mode waves moving in opposite directions along the $L=3.3$ flux tube. For gyroresonance on this flux tube at the equator, the parallel component of energy of the electrons is 25 keV at 3 kHz in the whistler band, or 100 keV at 1 kHz below it. A possible explanation is that energetic electrons of a sufficiently anisotropic pitch-angle distribution-function and associated with electrons injected during an earlier auroral substorm become unstable, through cyclotron resonance instability, when they drift into the high density plasmasphere. The instability causing the burst of electron precipitation was believed to be triggered by the half-hop whistler propagating across the equator. The whistler was amplified during the interaction; it was later observed, above the

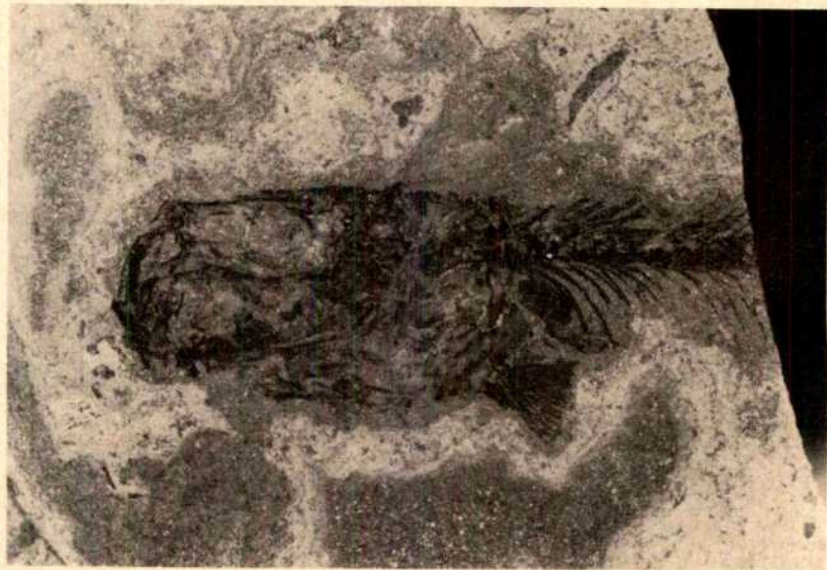
background noise level, as a two-hop whistler.

Voss *et al.* have measured this wave-particle interaction by sophisticated solid-state instrumentation cooled to 150 K aboard the S 81-1 satellite in a polar orbit at 230 km altitude. They show several examples of one-hop whistlers, observed on the ground in Antarctica, that are associated with bursts of precipitating electrons. The flux of precipitating electrons is observed to rise by a factor of up to 100 within 0.2 seconds. Because the cyclotron resonance interaction reduces the pitch angle of energetic electrons trapped in the magnetosphere, their mirror points descend below the satellite altitude. For cyclotron resonance in the equatorial plane of the $L=2.2 \pm 0.1$ flux tube, N is estimated to be 3200 cm^{-3} ; waves with a frequency of 4 kHz resonate with 150 keV electrons. Such energetic particles can penetrate the atmosphere down to a height of 80 km, where they cause a significant amount of secondary ionization. This can affect the characteristics of very low frequency radio waves propagating in the Earth-ionosphere waveguide.

The overall efficiency of whistlers in causing the loss of the van Allen radiation belt electrons is still not known. However, with a globally-averaged rate of about 100 lightning discharges per second (Brookes, C.E.P. *Geophys. Mem.*, London 24; 1925) there is considerable scope for further experimental studies of this wave-particle interaction. Such studies are also of relevance to other magnetospheres in the Universe, and to the loss of charged particles from plasma devices in any Earth-bound laboratory that relies on magnetic mirrors for plasma containment. □

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A CORE sample, obtained in the course of test-drilling from a North Sea oil rig belonging to Marathon Oil, produced this 150-million year old fossil of the front half of the fish *Tharsis dubius*, now in the British Museum of Natural History.



Tropical ecology

Long-term rainforest studies

from Jared M. Diamond

MOST biologists have their permanent homes in the temperate zones. The most species-rich biological communities are in the tropics, the rainforests of which are often described by stereotypes such as 'seasonally constant' and 'complex'. Dr. Harry Bell of the University of New England in Armidale, Australia is responsible for one of the few uninterrupted and intensive ecological studies by biologists resident in the tropics and his recent papers provide a wealth of quantitative information for tropical bird communities of New Guinea.

Following a series of studies that began in 1964, Bell devoted 1975–1978 to a census of the 165 bird species on a 2.5 hectare plot of rainforest^{1,2} and on a nearby 1-km transect across savanna³. He estimated the diet, body weight, population density, vertical distribution, and the seasonal changes in status of each species.

Although New Guinea is near the equator and 80 per cent of the rainforest bird species show no significant seasonal changes in abundance, their breeding is as markedly seasonal as in the temperate zones, although geared to changes in rainfall rather than day-length^{1,3,4}. Most carnivores and omnivores breed in the drier season, before the onset of the heavy rains that would damage their nests¹. Most ground-feeders breed in the wetter season, when the ground is soft and food more easily extracted^{1,4}. The grass warbler *Cisticola exilis* starts calling and displaying within three days of a single rain, and ceases again in a few days if there is no more rain³.

Compared with temperature-zone birds, those of New Guinea have low nesting success, low recruitment of young, and are long-lived^{1,4,5}. Open nests contain only 1.4 eggs on average (4–6 in temperate zones), and an astonishingly high number (88 per cent) of the clutches fail¹. Of young paradise kingfishers that leave the nest, most disperse, starve and die in their first year without having obtained a territory⁴. Conversely, annual mortality of adults is only 10–20 per cent⁵.

Bell estimates there to be 69 birds per hectare, between 3–30 times more than in most temperature-zone forests¹. Bird biomass is about 5 kg per hectare. Even compared with other tropical areas, New Guinea has a high species diversity and biomass of obligate frugivores (especially, large pigeons and parrots) — probably an evolutionary result of the paucity of fruit-eating mammals on the island.

Some of Bell's observations highlight two under-appreciated factors in habitat selection by rainforest species. First, some insectivores of the dark understory with

large eyes refuse to cross even small sunlit paths¹, whereas other species of the forest canopy seem to need strong sunlight. Second, the parrot *Pseudeos fuscata* will never cross a mountain pass through which a stream runs, in its daily commuting of up to 50 km from roosting to feeding sites across the lofty Central Cordillera of New Guinea¹.

Bell's papers abound with examples of the complex interspecies relations for which the tropics are famous. Kingfishers and pygmy parrots excavate their nests in arboreal termite nests^{4,6,7}. Different kingfisher species use different-sized nests, and the density of termite nests probably limits kingfisher abundance in second growth⁷. Ground pigeons that swallow pebbles, and thereby can grind up hard seeds in their stomachs, collect seeds defecated or regurgitated intact by frugivorous bird species with weak-walled stomachs⁶. The male and female of the flycatcher *Arses telescopthalmus* are each more similar ecologically to other fly-catcher species than to one another. Compared with the female, the male has longer and more curved claws and a more slender bill, for gleaning insects off understory vertical trunks and vines, whereas the female has a longer tail, wider

bill, and more rectal bristles, for catching insects in midair⁸.

One of the most interesting examples of group behaviour involves the babbler *Pomatostomus isidori*¹. This species lives in groups of about eight, which jointly build one nest, roost in the nest, and rear and feed one or two young. They forage together and exchange incessant contact calls and an all-clear 'yahoo' call, with one individual acting as leader and giving a distinctive 'leader call'. The babbler flock is followed by rusty pitohuis (*Pitohui ferrugineus*), and both the babblers and pitohuis are followed by bird species of several other families (including birds of paradise), all of which mimic the babbler's and pitohui's rusty colour, and some of which also mimic the calls.

Until recent decades, tropical ecology was regarded as hopelessly complex, and graduating ecologists were advised to study simpler communities like the desert and the arctic. Bell's papers set new standards for quantitative long-term tropical work and show that rainforest studies are complex and fascinating but not hopeless. □

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Biotechnology

Cancer toxin genes cloned

from Karol Sikora

CLINICAL research into cancer treatment badly needs new leads. The major stumbling block is the poor discrimination of systemic agents for common solid tumours. Some immunological mediators are known to have specific anti-tumour effects but the mechanisms involved are poorly understood. Ultimately, it is only through molecular cloning that the individual proteins will be characterized and their functions elucidated. Interferons, the first to be cloned, are now undergoing extensive, if rather disappointing, clinical trials¹. On page 721 and 724 of this issue the cloning of two other molecules of great clinical potential, lymphotoxin and tumour necrosis factor, is reported^{2,3}.

The discovery of cancer toxins dates back at least to 1891 when a New York physician, William Coley, began giving patients mixtures of toxins from various bacteria⁴. His rationale was that cancers sometimes regressed in patients after a severe bacterial infection. The dramatic tumour responses he saw were later reported with evangelical zeal⁵ and the whole sub-

ject of the anti-tumour effects of bacterial endotoxins heralded in the era of active immunotherapy. But the bacterial products themselves were not found to destroy tumour cells *in vitro*. This paradox was resolved by the discovery that endotoxins, when injected into suitably primed animals, stimulate macrophages to produce a 'tumour necrosis factor' (TNF) that causes haemorrhagic necrosis in tumours⁶. But even before this, tumour immunologists had demonstrated that sensitized lymphocytes could also destroy tumour cells through soluble mediators — the lymphokines, including lymphotoxin (LT)⁷.

Antibodies to partially purified TNF and LT provided evidence of structural differences between the two cancer toxins, both of which are able to distinguish between tumour cells and normal cells, and destroy only the former^{8,9}. But although an intriguing new pair of selective tumour-destroying agents had been discovered, experimentalists were plagued by their impurity. Batch-to-batch variation, tedious bioassays and the lack of molecular characterization precluded further analysis. Indeed,

the observations were dismissed by some as an over-optimistic interpretation of confused data.

Against the background of doubt, the use of sophisticated biotechnology has now unravelled the structure of both TNF and LT. The gene for TNF was cloned from the human promyelocytic leukaemia line HL60, which produces large quantities of TNF upon stimulation. Amino acid sequence data from tryptic peptide analysis led to the construction of a 42-base oligodeoxynucleotide that was used to extract TNF cDNA from a cDNA library. From the sequence of the cDNA it is clear that TNF is made as a 157-amino acid precursor from which the amino-terminal 76-amino acid segment is cleaved prior to secretion of TNF. The cleaved fragment may well represent an unusually long signal peptide and contains arginine and lysine dipetide pairs of the kind often associated with the release of physiologically-important peptides from precursor molecules¹⁰. The TNF produced when the cDNA was expressed in in *Escherichia coli* was tested for biological activity; tumour necrosis is demonstrable with microgramme quantities.

LT was purified from a phorbol ester-stimulated human lymphoblastoid cell line, its amino-terminal 155 amino acids were determined by microsequencing and a corresponding oligodeoxynucleotide was synthesized. This long probe was used to isolate a cDNA clone from which the complete LT sequence was derived. Again, a signal sequence is found, in this case 34 amino acids long. LT produced from the cDNA in *Escherichia coli* has clear anti-proliferative effects on several human tumour cell lines but not on normal cells. Furthermore, it destroyed a murine sarcoma *in vivo*.

Both molecules can kill tumour cells. Can we learn anything of their structure-activity relationships? It turns out that there is considerable similarity between the sequences of TNF and LT. Of the 157 amino acids of TNF, 44 are matched in LT when suitable gaps are introduced. Many more amino acids show conservative changes. There are two well conserved regions within the molecules in addition to the hydrophobic carboxy-termini. One conserved region perhaps binds to a cellular receptor, the others may effect cell destruction. Many intriguing questions can now be addressed. What is the basis for

selective tumour cell destruction? Do these toxins have to be released in the vicinity of a tumour or are they effective systemically? Is there species-specificity? Now that the genes are cloned, material will be available for these studies.

What of the clinical potential of the toxins? Several groups have cloned, or are on their way to cloning, the DNA for one or both toxins and clinical trials will start soon. Animal experiments are in progress to determine the pharmacokinetics of tumour destruction. Most of the early animal work used crude extracts injected into the tumours but the clinician will be more interested in systemic therapy

because of the widespread nature of metastatic disease. As with the interferons, there may be profound side effects¹¹. In fact, the symptoms of many diseases are probably mediated through the release by the immune system of proteins such as TNF and LT. The elucidation of the precise structure of these two molecules and their production in a pure form will stimulate renewed interest in their clinical exploitation and in the biological mechanisms that control cancer. □

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Molecular biology

Upstream and downstream control of eukaryotic genes

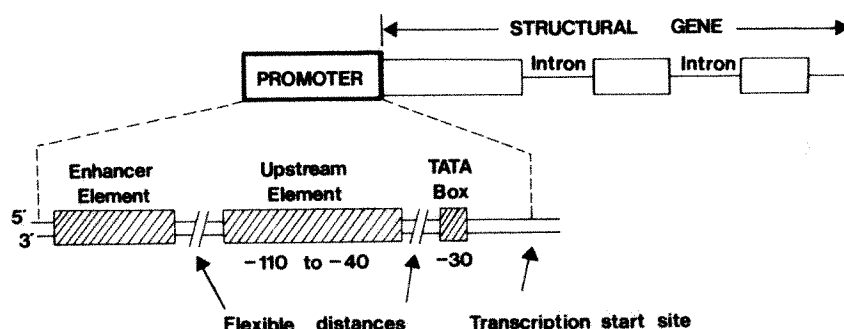
from Tim Reudelhuber

How does a specialized cell selectively express only a small subset of the tens of thousands of genes at its disposal? This question is of fundamental importance in molecular biology and may also have practical importance in such fields as medicine and agriculture. In prokaryotes, transcription is controlled by regions of DNA on the upstream, or 5', side of structural genes. These regulatory or promoter regions are composed of a set of DNA sequence elements whose function, in most cases, has been clearly demonstrated. As a logical extension of this knowledge, the attempt to identify transcriptional control elements in eukaryotes has also been focused, with considerable success, on the DNA sequences upstream of structural genes. But two recent reports in *Cell* suggest that the human adult β -globin gene may also contain a crucial control element downstream of its transcription start site^{1,2}.

The upstream control elements of eukaryotic genes are classified into three types based on their function, their sequence characteristics and their position relative to the start site of transcription (see Fig. and ref. 3). One type, termed a TATA box element (because the sequence is fre-

quently TATA), is involved in fixing the start site of transcription to a point 30 base pairs downstream from its own position. A second type, the upstream element, is a broad class of sequences, at a variable distance from the transcription start site, that seems to be important in determining the level of transcription. These elements have variously been referred to as G-C rich elements and even CCAAT boxes (again because of their sequence). Finally, enhancer elements, which were first reported in mammalian viruses, seem to be able to stimulate gene transcription from considerable distances and have been postulated to be tissue-specific modulators of transcription for some mammalian genes (see for example refs. 4, 5).

The evidence that a human globin gene is, in part, controlled by a downstream element arose from attempts to define the DNA sequences responsible for the differential expression of human α -globin and β -globin genes. The adult α -globin gene is expressed throughout fetal and post-natal life whereas the members of the β group are expressed during development in the order ϵ (embryonic), γ (fetal) and adult β globin. The controlling sequences for this differential expression were investigated by



Features of eukaryotic protein-coding genes. Protein-coding sequences (open boxes) and interspersed introns comprise the structural gene, upstream of which is a promoter region containing three types of control elements. (Courtesy A. Wildeman.)

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Charnay *et al.* by means of introducing the cloned genes into a mouse erythroleukemia (MEL) cell line¹. If terminal differentiation of MEL cells is induced by various agents *in vitro*, there is a concomitant activation of transcription from the endogenous α -globin and β -globin genes and from the introduced cloned human β -globin gene, but not from the introduced cloned human α -globin gene (which seems, inexplicably, to be expressed constitutively before and after differentiation).

The striking finding reported by Charnay *et al.*¹ is that a hybrid of the human α -globin upstream promoter region and the β -globin structural gene sequences is inducible in the same manner as the complete β -globin gene. An identical finding is reported by Wright *et al.* from experiments with hybrid genes that consist of fusions between the promoter of either the non-inducible human fetal (γ) globin gene or a mouse histocompatibility antigen (H-2K^{bml}) gene and human adult β -globin structural gene sequences. Again, upon introduction into MEL cells, the hybrid genes were β -globin-like in their induction. These results suggest that the β -globin gene, which has a full complement of functionally important upstream promoter elements⁶, also harbours an element within the structural gene that is crucial for appropriate expression in erythroid cells.

With these findings, the human β -globin gene joins a small but growing number of eukaryotic genes that have been shown to have regulatory sequences downstream from the start site of transcription. The *Xenopus* 5S ribosomal RNA gene (which is transcribed by a different RNA polymerase) also has a regulatory element within the domain of its structural gene⁷; some mouse immunoglobulin genes contain an enhancer element within an intron⁴; and the chicken thymidine kinase gene has an intragenic control element (although it is not yet known whether this control is at the transcriptional level)⁸.

Could gene regulation be one of the functions of the gene-splitting introns of eukaryotes? Circumstantial support for this hypothesis comes from the finding that the introns of many coordinately-controlled and neurone-specific genes in the rat brain contain some sequences in common⁹. In any case, it is apparent that despite the tremendous recent progress in determining the control elements of eukaryotic genes, we have still a lot to learn. □

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Endocrinology

Enlightenment and confusion over steroid hormone receptors

from R.J.B. King

STEROID hormones combine with specific receptor proteins to form a complex that binds to cell nuclei, thereby switching on messenger RNA (mRNA) synthesis from specific genes. That statement could have been made nearly twenty years ago; the intervening years have been spent in sorting out the nuts and bolts of the model. Major questions have been: what is the relationship of the cytoplasmic '8S' receptor protein (if it exists) to the smaller nuclear receptor; what is the nuclear acceptor for the hormone-receptor complex; how does the complex switch on the genes; and how do different hormones influence the same cellular function?

Many of the difficulties encountered in attempts to answer these questions can be ascribed to two practical problems. First, receptors have, until recently, only been detectable by the use of radioactive steroids, so that in most cases only the functionality of the steroid-binding domain of the receptor has been followed. Second, there has often been no certain way of testing the biological relevance of the molecular interactions being studied.

In the case of the glucocorticoid hormones, the latter problem has been partly resolved by the isolation of variant mouse lymphoma cell lines that are abnormally unresponsive to glucocorticoids¹. The variant cells fall into three categories: those that are deficient in the cytoplasmic receptor (r^-); those that are defective in nuclear transfer (nt^-); and those with increased nuclear transfer (nt^+). These variants have enabled some correlations between receptor properties and biological function to be made. Work on the glucocorticoid receptor has also been helped by the molecular dissection of mouse mammary tumour virus (MTV), which is transcribed into RNA when induced by glucocorticoids. A combination of studies involving the construction of chimaeric genes, a range of viral deletion mutants and the binding of purified receptor to specific DNA constructs has suggested that glucocorticoid receptor switches on MTV transcription by binding to specific sequences within the 'long terminal repeat' of MTV²⁻⁴. In other genes, too, the evidence is pointing to a region of DNA upstream of the mRNA initiation site as the acceptor site for steroid receptors. Interestingly, there are additional binding sites for the receptor. They are within the structural gene and of unknown function.

If all steroid receptors bind to some upstream gene region, it could explain how one biological function can be produced by different steroids through the appropriate

receptors. Glucocorticoid and progesterone receptors have recently been shown to bind *in vitro* to overlapping regions of the MTV and chicken lysozyme DNA^{6,7}, and there is increasing comment about consensus sequences able to recognize different receptors. Moreover, unpublished data from my own laboratory suggest that the long terminal repeat of MTV, which classically responds only to glucocorticoids, responds to androgens after it is experimentally transferred to cells that carry androgen receptors.

As to the shortcomings of relying on radioactive ligands, it has long been accepted wisdom that they would largely be overcome by the production of good antibodies to receptors. This has proved to be correct but only at the cost of raising both tempers and more questions. Where monoclonal antibodies have been generated unequivocally against the steroid-binding subunit of a receptor, lack of cross-reactivity with other classes of steroid receptor has been established and, in some cases, receptor genes and their mRNAs identified. Thus, on page 779 of this issue, the groups of Yamamoto and Gustafsson describe the use of MTV, the lymphoma variants and monoclonal antibodies to a glucocorticoid receptor, not only to identify the receptor gene and its mRNA but also to characterize further the lymphoma variants⁸. Perhaps their most interesting result is that even the r^- cells produce a normal-sized mRNA from the receptor gene, albeit in reduced quantity. Why should there be any mRNA in the r^- cells? The authors suggest that the r^- (and nt^+) cells produce a non-functional 'receptor' protein.

A 'receptor' that does not bind hormone — in this case progesterone — has also been identified in chick oviduct cells and its cDNA has recently been isolated in O'Malley's laboratory. A monoclonal antibody against the B-subunit of the receptor was used to identify the gene product. The antigen is similar to the progesterone-binding domain both with respect to size and peptide map. On the other hand, Baulieu's group has a monoclonal antibody to chick oviduct progesterone receptor which recognizes a protein that does not bind steroid, shows no similarity to the steroid-binding domains and which is claimed to be common to all classes of receptor¹⁰. O'Malley's group, however, has since suggested that this protein is an artefact¹¹.

Thus, there are at least three candidate components of the '8S' receptor — a steroid-binding subunit and two non-

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binding subunits one of which is similar, and the other dissimilar to the binding subunit. Additional examples exist of monoclonal antibodies raised against a partially purified glucocorticoid receptor¹² or an oestradiol receptor (in my own laboratory) that do not react with steroid binding subunits. Confusion abounds. But if the nuclear rather than cytoplasmic location of unoccupied oestradiol receptor is verified¹³, the '8S' receptor may anyway be an artefact.

My own feeling is that, given the impure nature of the receptor preparations used to raise the monoclonal antibodies and the general ability of steroid-binding subunits to react non-specifically with a wide range of macromolecules, the characterization of the antibodies should be closely scrutinized. If the antibody reacts directly and unequivocally with a steroid-binding protein, then valid deductions are in order; if this criterion is not satisfied, data from the system should be interpreted with caution. My current view is that the texts on receptor structure will require rewriting but it would be

foolhardy to start just yet. On the other hand, work on gene isolation of the type described in this issue of *Nature* represents a major advance towards our understanding of steroid hormone action. □

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Earth sciences

Suspect Sm-Nd whole-rock ages

from M.J. Bickle

WELL-preserved volcanic and sedimentary rocks in Archaean greenstone belts provide a unique record of surface and magmatic processes during the first half of the history of the Earth. Unravelling stratigraphic relationships in the structurally complex greenstone terrains is the first prerequisite to their interpretation. This depends largely on isotopic methods of geochronology, particularly the decay of uranium to lead and of samarium to neodymium in whole-rock samples. Although most Sm-Nd ages determined in whole-rock samples agree well with ages derived by other methods, some significant discrepancies have recently emerged, highlighted this year by reports of markedly different ages for the volcanic succession of Kambalda in Western Australia^{1,2}. The age of the Kambalda sequence is important not only because of its tectonic significance but also as a guide to the search for nickel ores.

For Kambalda, Claoué-Long *et al.*¹ obtain a whole-rock Sm-Nd isochron of $3,262 \pm 44$ Ma (2σ errors) on samples of komatiite, high-Mg basalt and tholeiitic basalt. This is about 400 Ma older than, both a Pb-Pb age of $2,720 \pm 105$ Ma on the same volcanic rocks and model Pb ages of $2,840 \pm 35$ Ma on the associated iron-nickel-sulphide ores obtained by Roddick². Until recently, the Sm-Nd age would have been accepted without question because it satisfies the normal criteria. In particular, the samples include only basalts and magnesium-rich komatiite lavas, and not the felsic volcanics and

intrusives that may not be cogenetic but were included in a previous study³.

U-Pb systematics in volcanic rocks are relatively easily altered and, as discussed by Roddick², the Pb-Pb whole-rock age on the volcanics may be reset by metamorphism. The model Pb ages of $2,840 \pm 35$ Ma on the iron-nickel-sulphide ores are less easy to reconcile with the Sm-Nd age. Field, petrological and geochemical criteria⁴ are consistent with separation of the iron-nickel-sulphide ores as an immiscible sulphide liquid during emplacement of the high-temperature komatiite lavas; alteration of the Pb isotopic compositions of 3,260 Ma Pb-rich, U-poor sulphide ore to lie near the 2,800 Ma point on the geochron is not easy to envisage.

A major difficulty is that the age discrepancy is not readily testable by alternative methods as there are few independent constraints on the age of the Kambalda sequence. The only good date available from the Kambalda area is a minimum age set by an intrusive $2,820 \pm 15$ Ma granodiorite⁵. Stratigraphic correlation of the sequence at Kambalda with surrounding areas is uncertain in this structurally complex and poorly exposed terrain; indeed, this is an area where geologists wish to use isotope geochronology to test the stratigraphy.

The only piece of evidence seriously to challenge the Sm-Nd result is the Pb isotopic composition of the iron-nickel-sulphide ore, but model Pb age calculations are essentially empirical and do not

provide internal tests of consistency. Even so, may the Sm-Nd be in error? A few recent studies have revealed potential problems with Sm-Nd geochronology that may be partially explained by the emplacement of high-temperature komatiite lavas. A sequence of basalts and komatiites in the Abitibi Belt, Canada, gives a whole-rock Sm-Nd isochron of $2,826 \pm 64$ Ma, but the age of the rocks is well constrained to less than $2,697 \pm 1.1$ Ma by a U-Pb zircon age on stratigraphically-underlying felsic volcanics⁶, suggesting that this systematic error in the Sm-Nd age results from heterogeneity of initial Nd isotopic ratios of the source⁶. Dupré *et al.*⁷ record variable Sm/Nd ratios within an individual flow elsewhere in the Abitibi Belt and conclude that the variation results from contamination due to melting and assimilation of the underlying flow. Thus, both source heterogeneity and contamination could lead to systematic errors in Sm-Nd whole-rock ages.

Where the initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratio and Sm/Nd ratio correlate, a linear relationship between the two parameters will be preserved on the isochron diagram but age calculation will be systematically in error. Correlation of any initial daughter isotope ratio with parent/daughter ratio may occur in any whole-rock geochronological method, but the very similar geochemical behaviour of the rare-earth Sm and Nd limits the dispersion of Sm/Nd ratios and makes the age calculations sensitive to small systematic differences in initial Nd isotopic composition. Both mantle source heterogeneity and contamination can lead to initial $^{143}\text{Nd}/^{144}\text{Nd}$ and Sm/Nd ratios that are relatively well correlated.

If the 3,260 Ma age of the Kambalda suite is a result of source heterogeneity, the mantle source regions for the lavas must have had a long history of variable depletion by 2,800 Ma. Whether petrogenetic processes of melting and fractionation could erupt lavas with unchanged, or systematically changed, Sm/Nd ratios is open to question.

The likelihood of contamination of the unusually high temperature komatiite lavas has been given new emphasis by recent theoretical and experimental modelling of their emplacement. Huppert *et al.*⁸ have suggested that the flows may melt up to 10 per cent of their volume of underlying rock. This process may be the cause of the deep channels at Kambalda, which are cut into the underlying basalt and

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filled with iron-nickel-sulphide and komatiite⁹. Similar contamination by melting wall rock during ascent of magmas through the crust is equally likely.

Until criteria to choose between mantle heterogeneity contamination or complete resetting of the Pb isotopes are found, or an independent age constraint is available on Kambalda, the uncertainty in the interpretation of the Sm-Nd age will remain. Resolution of the problem may have

important implications either for the degree of heterogeneity and depletion of the Archaean mantle or the extent of contamination of komatiitic lavas. The latter may necessitate reinterpretation of the constraints komatiite lavas place on the chemistry of the Archaean mantle. □

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Ecology

Why be an evergreen?

from Peter D. Moore

CONSIDERING their importance to plants, it is surprising that we do not know more about leaves. What, for example, determines leaf size and what is the adaptive significance of size and longevity in leaves? Presumably the evolution of the evergreen habit conveys certain advantages upon a plant under given environmental conditions. What they may be is the question raised by three new studies¹⁻³.

It has long been recognized that there is a relationship between leaf form and climate⁴ but the causes underlying the relationship have proved elusive. Parkhurst and Loucks⁵ constructed a model of optimal leaf size on the assumption that the optimum under given conditions is that which provides the maximum efficiency of water use, that is the maximum uptake of CO₂ per unit of water lost. Thus, large leaves should be favoured only under warm conditions with low light intensity. They tested their model, which accounted for seven independent variables, and found it to match well with observed data. It has subsequently been shown to operate in a variety of situations including the Galapagos Islands, where there is a positive relationship between the average leaf size of a plant community and rainfall, and where leaves in the herbaceous layer of a structured community are larger when the canopy layer is denser⁶.

Although leaf size has been shown to follow certain rules, at least on a large scale consideration, leaf longevity has proved less amenable to explanation. Hamann found it difficult to explain the distribution pattern of evergreen species on the Galapagos Islands; no advantages over their deciduous counterparts were immediately apparent, though evergreens appear to be more frequent in sites with more prolonged drought⁶.

Explanations of the advantages of the evergreen habit have tended to fall into two camps. The main advantage is claimed to be energetic, the evergreen leaf being capable of taking advantage of short periods of suitable conditions within a generally unfavourable period of, for example, cold or drought. The alternative explanation proposes that long-lived

evergreen leaves act as storage systems for nutrients in short supply, such as nitrogen, phosphorus and potassium⁷, and that it is therefore of selective advantage to be an evergreen in low nutrient environments such as bogs and heathland.

In their recent review of the energetic advantages of evergreens in winter conditions, Larcher and Bauer⁸ comment on the scarcity of data. In general they feel that winter photosynthesis may be significant in Mediterranean and oceanic areas but it unlikely to be of importance in regions with severe winters. Robertson and Woolhouse¹ have now shown that a herbaceous species, the cotton sedge *Eriophorum vaginatum*, a major dominant species on the upland areas of northern and central Europe, retains some leaves through the winter which are capable of activity very early in the growing season. These leaves can be operating at up to half-maximal rates before the new season's crop of leaves has been produced, and they continue to photosynthesize throughout the growing season. Interestingly, however, the leaves of *E. vaginatum* also act as reservoirs for elements such as phosphorus and potassium⁹, so a combination of advantages could be involved here.

These observations apply to oceanic

environments but some comparable data have emerged from a distinctly continental environment in Utah in the United States. Nowak and Caldwell² monitored the photosynthetic rates of two introduced grasses, *Agropyron desertorum* and *A. spicatum*, during winter when the soil is permanently frozen and snow cover is intermittent. Many leaves of these grasses survive the winter and show a positive carbon gain during those periods when they are not covered with snow. Such observations provide fuel for the energetic advantages of evergreen leaves.

At the other end of the climate spectrum are the forests of north-east India. Shukla and Ramakrishnan find that the majority of early successional trees are of evergreen habit, but that the leaves are generally short-lived (usually less than 200 days) whereas late successional species, have a deciduous habit and their leaves may last 300 days. In that case, evergreen habit could be of advantage in providing fast growth, especially when combined with a rapid leaf turnover, enabling new leaves to be produced in the most appropriate positions to take advantage of the light.

The impression gained from these varied studies in a range of climatic regimes is that there is no simple, general reason why the evergreen habit can be of selective advantage. Both nutrient conservation and energetic opportunism may give a plant the necessary competitive edge when facing an unfavourable period. It clearly pays some plants to keep their leaves. □

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100 year ago

PRONUNCIATION OF CHINESE NAMES

SOMEWHAT after date, I beg to return to the subject of Anglo- and Franco-Chinese orthography, referred to in *NATURE* vol. xxx, p. 592. In a short paper of mine published in the *Proceedings of the Royal Geographical Society*, vol. xxii, No. 6, 1877, I alluded to the desirability of a uniform or fixed "Roman equivalent" for Chinese characters standing for names of place, &c. To my mind the Italian vowels, &c. come nearest to the sounds of the Chinese characters. *Tung-King*, meaning "Eastern Capital," is the usually accepted form of *Tonquin*, or *Ton-Kin*, the terminal g being

but slightly sounded. *Shang-hai*, the "Upper Sea," or the place "of going up to the sea," should be pronounced with the g, and is so spoken (Shanghai) by English and American authorities. Dr. Wells Williams has, I believe, in manuscript a standard Chinese Gazetteer of the world, in which all proper names likely to be used in telegraphy, newspapers, &c., are smoothly transliterated into Chinese characters. For translation from Chinese it is very necessary to adopt some such plan as Dr. Hunter has suggested for Indian names. Although his plan has come too late into the field to induce people to spell Calcutta as Kolkata, this is hardly the case as yet with Chinese names. The old native names of places should always be literally preserved. How much more beautiful is the old Franco-Indian name Stadacona than Quebec for the scene of death of Wolfe! I should be glad to co-operate or correspond with any interested in this matter, so prominent and important at the present juncture.

From *Nature* 31, 173, 25 December 1884.

Nuclear physics

Widening scope for new heavy-ion accelerators

from Shigeru Kubono

A NEW generation of heavy-ion accelerators is opening up fresh fields of research in the natural sciences — including astrophysics, solid state physics, medicine and geology — as well as finding more conventional application in nuclear physics. One of their biggest successes so far has been the production of new elements, heavier than ever seen before in nuclear reactions. An element of atomic number (Z) = 108 has recently been synthesized at GSI Darmstadt in West Germany following the discovery of $Z = 106$ and $Z = 109$ elements, and experiments have been planned to search for elements of $Z = 110$ and heavier. These and other new findings in nuclear physics, along with applications of heavy-ion beams in other areas, were the theme of a two-part symposium* held in Japan.

Since the Second World War, light-ion and electron beams have been used extensively to investigate nuclear structure and reaction mechanisms. Heavy-ion beams, with energies up to 10 MeV per nucleon, made their appearance in the 1960s. Several new generation machines, now with energies of ten to a few hundred MeV per nucleon, have already been completed — principally at Michigan State University, Caen in France (GANIL), and Darmstadt (GSI) — and several more are on their way, including RIKEN in Japan and at Lanzhou in China.

The new heavy-ion beams have a wide range of application in the natural sciences. They are highly effective in cancer radiotherapy, as shown in a recent joint US-Japan cooperative research project (K. Sakamoto, Tohoku University). The main advantages of such beams over other particle beams are the higher efficiency with which they kill cells, the independence of their cell-killing from the stage of the cell cycle and their selectivity for killing tumour cells rather than normal cells. So far, only the Lawrence Berkeley Laboratory has radiotherapy facilities; experiments with carbon, neon and silicon ions have been tried.

Transfer of energy from heavy ions causes a variety of effects at solid surfaces and interfaces. These are the basis of a number of applications that are likely to be of great commercial value, including the reduction of surface reflectivity in scintillators, improvement of thin film adhesion and the establishment of electrical con-

tacts, and the implantation of ions into semiconductors (T. Tombrello, California Institute of Technology).

Heavy-ion beams can also be used in mass spectrometry, allowing detection of isotope ratios as small as 10^{-14} . In addition to various archaeological applications, this will make it possible to use neutrino detectors such as ^{71}Ge or ^{205}Tl rather than conventionally used ^{37}Cl . These detectors would provide a good sensitivity for neutrinos from the earliest stage of the pp chain in stellar evolution.

Several quite new problems in nuclear physics can also be tackled with the aid of the new accelerators. One proposal (E. Adelberger, University of Washington, Seattle) is to look for charge symmetry breakdown in the very high excitation energy region of nuclei by comparing heavy ion- and light ion-induced reactions. Adelberger also suggests a search for breakdown of the time-reversal symmetry by using the elastic scattering of polarized neutrons from certain heavy nuclei. Other important topics that can now be tackled experimentally are the production of high-density nuclear matter in relativistic heavy-ion scattering (S. Nagamiya, University of Tokyo) and the important role that may be played by very neutron-rich nuclei in the early stage of the Universe (K. Sato, University of Tokyo).

Quantum electrodynamics has been tested by looking at the decay of the vacuum by the spontaneous positron emission in the supercritical fields of a giant nuclear system of high atomic number. In the presence of the very strong electric fields produced by such systems the vacuum is predicted to decay by positron emission. Such a phenomenon has been observed experimentally at GSI and the result shown to be compatible with theory (B. Müller, J. Goethe University, Frankfurt). The phenomenon appears to follow the production of a giant nuclear system of $Z = 173$ –188.

Conventional nuclear physics has also received a boost from the new heavy-ion accelerators. In addition to the discovery of the $Z = 108$ element and plans for the search for even heavier elements and nuclei that are far off the stability line on the nuclear chart (G. Münzerberg and O. Klepper, GSI), giant resonances — particularly high energy octupole resonances — have been shown to be better excited by heavy ions (D. Youngblood, Texas A. & M. University). Some remarkable new detectors were also described. One is a 'crystal ball' that covers almost all the space around the target where the nuclear

reaction takes place with a NaI or BGO crystal. Studies of very high spin states and structure have made tremendous progress with the help of such a detector (J. Garrett, Niels Bohr Institute), as have studies of the decay property of giant resonances (J. Beene, ORNL, USA) and of quasi-molecular resonances (V. Metag, University of Giessen, FRG).

Quasi-molecular states seem to have gained recognition as one of the general features of nuclei. A rotational band based on the quasi-molecular configuration now seems quite likely to exist below the threshold energy (S. Kubono, University of Tokyo) and even in very heavy nuclei (M. Gai, Yale University). A new reaction mechanism has been proposed in which fission does not go through compound nucleus formation; this process becomes more prominent as the incident energy of the heavy-ion beam increases (B. B. Back, Argonne National Laboratory). A time-dependent Hartree-Fock calculation for heavy-ion scattering has been used to predict that two colliding nuclei with low angular momenta will not fuse under some conditions. Possible evidence of such a low angular momentum cut-off has been measured (T. Nomura, RIKEN).

One of the most controversial subjects discussed at the symposia concerned absolute fusion cross-sections below the Coulomb barrier — a subject related to the proton-proton chain in stellar evolution (T. Kajino, Tokyo Metropolitan University). According to some reports, a contribution from quasi-elastic scattering processes is very important in reproducing the absolute values (T. Tamura, University of Texas; C. Signorini, Padova; N. Takigawa, Tohoku University); but an alternative explanation involves a process of neck formation (A. Iwamoto, JAERI).

As the incident energy increases, the reaction mechanism of heavy-ion scattering seems to evolve dramatically (C. Détraz, GANIL; R. G. Stokstad, Lawrence Berkeley Laboratory). The cross-sections of fusion reactions decrease in a systematic manner (S. M. Lee, University of Tsukuba; S. Harar, SACLAY) and new reaction mechanisms, such as break-up/fusion and fragmentation processes (H. Utsunomiya, Michigan State University; D. Gross, HMI; E. Rehm, Argonne National Laboratory), and a pre-equilibrium process (C. K. Gelbke, Michigan State University), start to play an important role. Another interesting subject in nucleus-nucleus scattering at an intermediate incident energy is the absolute cross-sections for pion production near the absolute threshold energy. The existing theories underestimate the π^0 production cross-sections by more than an order of magnitude. Why they do so remains an open question. □

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*The 1984 INS-ROKEN International Symposium on Heavy Ion Physics was held in two parts: 'New Scope of Heavy Ion Science', Tokyo, 24–25 August, 1984, and 'Heavy Ion Nuclear Physics', Mt Fuji, 27–31 August, 1984. The proceedings will be published in Supplements to the *Journal of the Physical Society of Japan*.

Epstein-Barr virus and carcinoma

SIR — A News and Views article by Rickinson¹ raises very important questions on the biology of Epstein-Barr virus (EBV). Since our report² that EBV genomes are regularly present in the epithelial cells of nasopharyngeal carcinoma (NPC), the discrepancy between *in vitro* experiments that failed to detect EBV receptors and the *in vivo* findings has been an important point of discussion. Rickinson discusses experiments by Sixby *et al.*^{3,4} on the detection of EBV genomes in exfoliated epithelial cells from throat washings and the finding that EBV can infect epithelial cells only if the virus is derived from throat washings, and not from tissue culture.

The data are interesting but not necessarily convincing. Sixby *et al.* have no evidence that the EBV-containing cells really originate from the throat, and their demonstration of the infection of epithelial cells involves an undefined system: throat washings contain a wide variety of components, including viruses of different kinds. Thus one must question their conclusion that mutants of EBV which are infectious to epithelial cells regularly exist or evolve. This would imply a very efficient and directed mutation of isolates of EBV obtained by collecting cell lines established from umbilical cord blood lymphocytes by immortalization with EBV from throat washings. The observation by Sixby may merely confirm the findings of Kehlifa and Menezes⁵, who were able to infect non-natural target cells of EBV (EBV receptor-free cells) by preinfection or simultaneous infection with Sendai viruses. The samples used by Sixby *et al.* may have contained pseudotypes of EBV particles and other viruses infectious for epithelial cells⁶.

Our recent data, published too late for Rickinson to have been aware of, suggests an alternative interpretation of Sixby's data. We have found that EBV can replicate in the parotid gland of patients lacking antibodies to major EBV-related early antigens. This site of replication allows lifelong production of EBV without effective stimulation of the humoral antibody response because the EBV-producing cells are in the lumen of the salivary duct and not in contact with the bloodstream. Perhaps these cells are shed with the saliva and are then detected in the throat. The virus released may in fact be responsible for a constant supply by new infection of peripheral B lymphocytes with EBV genomes. Rickinson also mentions the production by Yiwan *et al.* of an EBV-producing cell line after fusion of Tupaia cells with lymphoblastoid cells⁸. This may be a model for the establishment of persistent low level lytic cycles of EBV in specific cells unable to suppress EBV replication in the way peripheral lymphocytes from convalescents seemingly do.

Besides lytic infection, EBV is characterized by its ability to undergo

latency. It has long been known that in Burkitt's lymphoma and NPC biopsies, virus particles are only synthesized after one or two days of *in vitro* cultivation. As virus replication invariably leads to the lysis of the host cells, latency is a condition for a potential DNA tumour virus. In the case of NPC, the tumour cells are epithelial. The EBV genomes may enter these cells by mechanisms discussed above. However, mixed infections are very likely to kill the target cells. In addition, cells that can be infected by this mechanism should be located at the outer layers of the body and are therefore likely to be cells that have completed differentiation and are unable to live much longer. Aspirates of cells from the mucosa may contain such cells which may contain viral genomes but will even so undergo lysis⁹.

This does not mean that any transfer of EBV genomes to epithelial cells is an irreversible event leading invariably to the development of NPC. Nevertheless, the partial activation of EBV genes in lymphocytes harbouring otherwise latent EBV genomes and the consequent transfer of EBV genomes via EBV-mediated cell fusion to EBV receptor-negative epithelial cells¹⁰ would be a simple explanation for the presence of EBV genomes in submucosal epithelial cells. This mechanism would also offer a mechanistic explanation for the postulated activity of environmental agents as risk factors for the development of NPC.

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Succession theory is too reductionist

SIR — Bryan Finegan (*Nature* **312**, 109-114; 1984) is right to point out that the pendulum of succession theory has swung too far from the holist view to the reductionist, and his article is welcome. Any attempt to categorize all pioneer species in terms of *r*-selection would be perverse, bearing in mind that the heterogeneous nature of the environments that pioneers colonize — from open water through various bare rock materials (sand, gravel, lava) to mature soils. The ability of textbooks to quote successively the 'standard succession', which always starts with lichens, and the *r*-selected char-

acteristics of pioneers, has long puzzled me.

I would like to add two points. First, although he sensibly allows the mechanisms of facilitation, tolerance and inhibition to interact and occur throughout succession, there is a real sense in which facilitation is a first requirement.

Many, if not all, primary successions exhibit a qualitative shift at some stage — from aquatic to terrestrial, nitrogen-free to nitrogen-sufficient, very low to adequate water-holding capacity. These shifts determine whether late-successional species can grow in the initial stages — oak trees do not grow in water — and are achieved typically by autogenic change. In other words, facilitation is a widespread event in the early stages of primary succession because: (1) Species vary in their ability to grow in typical primary succession environments; and (2) the act of growing in such environments induces modification (terrestrialization, N and organic matter inputs). After the shift has been achieved, however, other processes may be much more important.

Second, I think that it is important to point out that not all macronutrients are deficient in early primary succession. Because of the lack of mineral N source in most soils, N is almost always limiting but P and K are almost entirely soil-derived. K is always entirely inorganic in soil, and may increase in availability due to inputs (particularly in maritime environments) and decomposition of minerals and decrease due to leaching and particulate loss. The overall trend is typically downwards. P is initially wholly inorganic, but over time declines in absolute amount, due to particulate loss and to a lesser extent leaching, while shifting gradually to organic forms. Most of the organic P cycles very slowly, if at all, and the solution P concentrations decline.

The net result of an increase in N availability and a decrease in P and K supply should produce a brief period, some time after the initiation of a succession, when productivity is maximal. Interestingly, such a pattern is frequently observed, though not usually ascribed to such a cause.

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Disease and the elm decline

SIR — I read the note by Peter Moore¹ on the historical decline of elms with considerable interest. I pointed out elsewhere² that the presence of elm bark beetle (*Scolytus scolytus*) remains in the fossil record does not prove that Dutch elm disease occurred, as the beetle may not always carry the fungal pathogen responsible for the disease. The only effective means of testing the disease hypothesis is to try to grow any

resting spores of *Ceratocystis ulmi* which may be found in fossil lake sediments in culture. Spores of other fungi from sediments as old as the Tertiary have hitherto been cultured successfully^{3,4}, so viability may not be a problem.

As cereal-type pollen has been shown to pre-date the elm decline^{5,6}, the real problem of interest to palaeoecologists now is probably not so much why the elm declined but how to find corroborative evidence for early agriculture. I suggest that a study of grass silica remains (phytoliths) could provide the answer. These may be washed into lakes from cropped areas and become incorporated in the sediments. Recent work from the United States^{7,8} indicates that the careful, detailed study of phytoliths can provide rather more palaeo-environmental information than was once supposed. Some papers on British phytoliths have been published⁹⁻¹¹ and these provide a foundation from which research could start. Additionally, it may be easier to determine phytoliths than pollen using automated methods, as the number of morphological types which occur is that much lower and their overall structures are simpler. It is always possible that identifiable portions of leaf cuticle, and not just phytoliths deriving from cuticles, can be found in fossil contexts and analysis of these might make research easier.

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Protein antigenicity and protein mobility

SIR — In an article in *Nature*, Westhof *et al.*¹ discuss the proposition that the antigenic determinants of proteins could belong to regions which show high mobility. In developing this correlation they have inadvertently overlooked work by ourselves². Using NMR methods which detect relatively slow motions as well as the fast motions seen in crystals, we observed that "the region of cytochromes *c* around Ile-57 is very flexible and it is striking that the best characterized antigenic site involves amino acid residues 58-62" (ref. 3).

We go on to state that for myoglobin and lysozyme, protein flexibility in charged surface segments is also important for antigenicity.

While not wishing to detract from the work of Westhof *et al.*, we think it right that attention should be drawn to this earlier work, since in the Oxford Enzyme Group we are engaged in a very detailed examination of mobility in relation to antigenicity in the cases of both cytochromes *c* and lysozyme. These studies, using both NMR⁴ and crystallographic methods to analyse mobility, show that the idea that such a correlation exists is confirmed but that mobility must be considered in the broadest context of motions on the slow time scale, perhaps even down to seconds, as well as at $<10^{-9}$ s. This means, of course, that the antigenicity need not be due to the ground state conformation or its mobility but can be related to a conformation of slightly higher energy⁵. Such states will be missed unless both crystallographic and NMR methods are used together.

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Doubts over the Colorado Plateau

SIR — I was puzzled by your comment in a News and Views leading article¹ on calderas "Can anyone now doubt that the Colorado Plateau . . . is a magmatic structure of the same kind?" Frankly, yes! I am wondering if it was Dan McKenzie's letter to *Nature*² which triggered your suggestion. If so, I think that you read something into his discussion that he did not mean to be there. He was speculating on the possible effects of injecting very large amounts of magma (complete provinces) into or beneath the crust throughout regions such as the Colorado Plateau, in order to account for their uplift without any internal deformation. Calderas are related to single volcanic centres and, although some of them are uncomfortably large (from the volcanic hazard point of view), they are all an order of magnitude smaller than the sort of multicentre regional phenomena that McKenzie was considering. Similarly, resurgent doming is a phenomenon of individual volcanic centres, rather than entire provinces. It is (as the name implies) a dome-like uplift, which may be related approximately to a point pressure source, as magma reoccupies the reservoir below. In contrast, the Colorado Plateau has risen like a lift floor, with essentially all the deformation around its margins. The Flat Tops area, in north-west Colorado, shows a similar style of uplift on a smaller scale than the plateau;

strata are flat-lying throughout its uplifted area but almost vertical along the monocline at its western margin.

I think that McKenzie's explanation of the plateau's uplift is probably correct. The region escaped the thermo-tectonic events which affected most of the western United States during the Mesozoic. At about 25 Myr, it was penetrated by scattered, explosive, low-volume, alkaline magmatism which was very loosely related to kimberlite. This carried up lithospheric xenoliths of garnet-lherzolite from depths as great as about 140 km (similar to South Africa), and seems to have originated from the top of the asthenosphere³. Subsequently, there have been large amounts of magmatism all around the plateau but virtually none upon it. The surrounding magmatism is compositionally essentially the same as that which characterizes ocean islands, except for local input from crustal fusion in areas of intense volcanic activity. If such liquids come ultimately from the asthenosphere (as must clearly be the case in ocean basins), then the existence and nature of asthenosphere beneath the plateau becomes the vital question. It appears to be both present at >140 km, and seismically normal^{4,5}. Thence, following the McKenzie model, one deduces that abundant magmas have left the asthenosphere beneath the plateau during the late Tertiary but none has reached the surface. The only point of McKenzie's analysis that I doubt concerns heat flow. This seems rather too low on the plateau to accommodate the massive amounts of geologically-recent intra-crustal and Moho-depth magmatism he envisages. Perhaps the uprising liquids consolidated deeper, within the sub-Plateau lithospheric mantle. Here they would ultimately become an episode of "mantle metasomatism", of the type (one of the types) that Hawkesworth and his colleagues study⁶.

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Mouse Igx sequence elements in *Drosophila* (erratum)

A letter under the above title from P. A. Tsonis appeared in Scientific Correspondence in the 22 November issue (*Nature* 312, 314; 1984) with the author's name omitted. □

Interstellar scattering in the inner parts of the Galaxy

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A new survey of the galactic plane for sources with size less than 1 arc s at 327 MHz shows that towards the inner parts of the galaxy for galactic latitudes less than 5°, interstellar scattering is much larger than expected from data at higher latitudes. The enhanced scattering varies both with galactic latitude and longitude. A two-component model for the distribution of scattering matter in the Galaxy is proposed to interpret the observations.

THE properties of the ionized component of the interstellar medium can be inferred from their effects on radio waves propagating through the medium. Scattering of radio waves by density inhomogeneities in the interstellar medium produces a number of observable effects, such as angular broadening of extragalactic sources, interstellar scintillation and temporal broadening of pulsar signals. Radio observations of pulsars have provided information on the properties of the local ionized interstellar medium. However, pulsars are not very effective probes for studying the large-scale properties of the interstellar medium because they have relatively low intrinsic luminosities; the estimated distance to the more distant pulsars may not be reliable. Although angular broadening of extragalactic radio sources does not have these limitations, the magnitude of this effect is so small (<1 arc s) that it has only been studied by interplanetary scintillation (IPS) and very long baseline interferometry (VLBI). At metre wavelengths, IPS is very useful for detecting and estimating the angular sizes of compact components in radio sources as all that is required is a large collecting area¹.

The simplicity of the IPS technique compared with VLBI makes it suitable for studies covering a large number of sources; however, the only existing survey² is restricted to the northern sky and hence provides no information on the scattering strength in the inner parts of the Galaxy. Therefore, we have used the Ooty Radio Telescope (ORT)³, which extends south to a declination limit of -35° , to perform at 327 MHz a new IPS survey, which includes the galactic centre. We find that interstellar scattering (ISS) is larger in the inner parts of our Galaxy and infer the variation of scattering strength with distance from the galactic centre.

Observations

We have studied two regions of the sky defined by

$$(A) \quad |b| < 10^\circ \quad \text{and} \quad -35^\circ < \delta < 0^\circ \quad (-10^\circ < l < 35^\circ)$$

$$(B) \quad |b| < 10^\circ \quad \text{and} \quad 5^\circ < \delta < 30^\circ \quad (170^\circ < l < 210^\circ)$$

where δ = declination, b = galactic latitude and l = galactic longitude. Catalogues of extragalactic radio sources at low galactic latitudes are confused by strong galactic sources; we have made, therefore, an unbiased IPS survey of all scintillating sources in regions A and B whether or not they are catalogued. We made drift observations when these regions were 10 – 25° from the Sun. With the 12-beam system of the ORT, we could observe a field of 36 sec δ arc min in declination; each part of the sky was in our beam for 8 min. Scintillating sources were detected from analogue records by their characteristic rapid variations. In addition, the mean and root mean square (r.m.s.) of the recorded data were plotted and examined for scintillating sources appearing as regions of enhanced r.m.s. By requiring that the variation of the r.m.s. of any genuine scintillating source should have the characteristic east–west and north–south beam-

shape of the ORT, we discriminate against terrestrial and solar interference and also confusion from the enhanced background temperature in the galactic plane. An IPS survey of the type described here is free from such confusion because the strong galactic sources are extended and therefore do not scintillate; pulsars are the only galactic sources that would be detected. Power spectral analysis of the recorded data enables us to identify pulsars with visible pulses at 327 MHz, but short period heavily scattered pulsars may exist, appearing as continuum sources. We repeated our observations on all regions that showed possible IPS.

Catalogues of small diameter radio sources in galactic centre region A have been made at 408 MHz for $|b| < 3^\circ$ (ref. 4) and $|b| > 3^\circ$ (ref. 5) at a sensitivity of about 0.7 Jy. All the sources for which we have reliable IPS detection could be identified with sources in these catalogues. However, it is possible that some genuine compact sources have been missed because of interference and telescope failures. To minimize this, we made IPS observations of all sources catalogued in ref. 4 and all those with total flux at 408 MHz > 0.8 Jy catalogued in ref. 5. We observed each source for 5–8 min at the optimal distance from the Sun to determine whether or not it showed IPS. However, we did not survey known sources in the anticentre region.

Sources with compact components were observed subsequently for 15 min each on 5–10 different days at different angular distances from the Sun. The equivalent half-power gaussian diameters were determined from the width of the power spectra of the intensity fluctuations, as measured by the second moment of the power spectra⁶. The required estimation of the interplanetary medium scattering parameters was made from repeated observations of the flat spectrum source 1148–001, whose angular size at 408 MHz is less than 0.015 arc s (ref. 7). A mean solar wind velocity of 400 km s^{-1} has been assumed.

For high galactic latitudes and IPS observations near the Sun, the system temperature of the ORT is about 300 K; with a collecting area of $8,000 \text{ m}^2$ and bandwidth of 4 MHz we can detect a source with scintillating flux (ΔS) around 60 mJy in 5 min. The system temperature of the ORT does not increase by more than 4 dB, apart from a $2^\circ \times 2^\circ$ region around the galactic centre, when the antenna is pointed at the galactic plane. In the anticentre direction the increase in system temperature is negligible except in the neighbourhood of the Crab Nebula. To have a uniform sensitivity limit for our entire survey, we have included only those sources which have $\Delta S \geq 0.2$ Jy. This conservative limit allows us to be confident that our survey is complete to this level of flux in region A. The survey of region B is incomplete; in the limited time available we concentrated on low-latitude regions.

Although our survey has a uniform sensitivity limit of $\Delta S = 0.2$ Jy, the sensitivity limit for the flux in the scintillating component (S_s), varies both with the ecliptic latitude and the angular diameter of the source. At 327 MHz, for angular distances

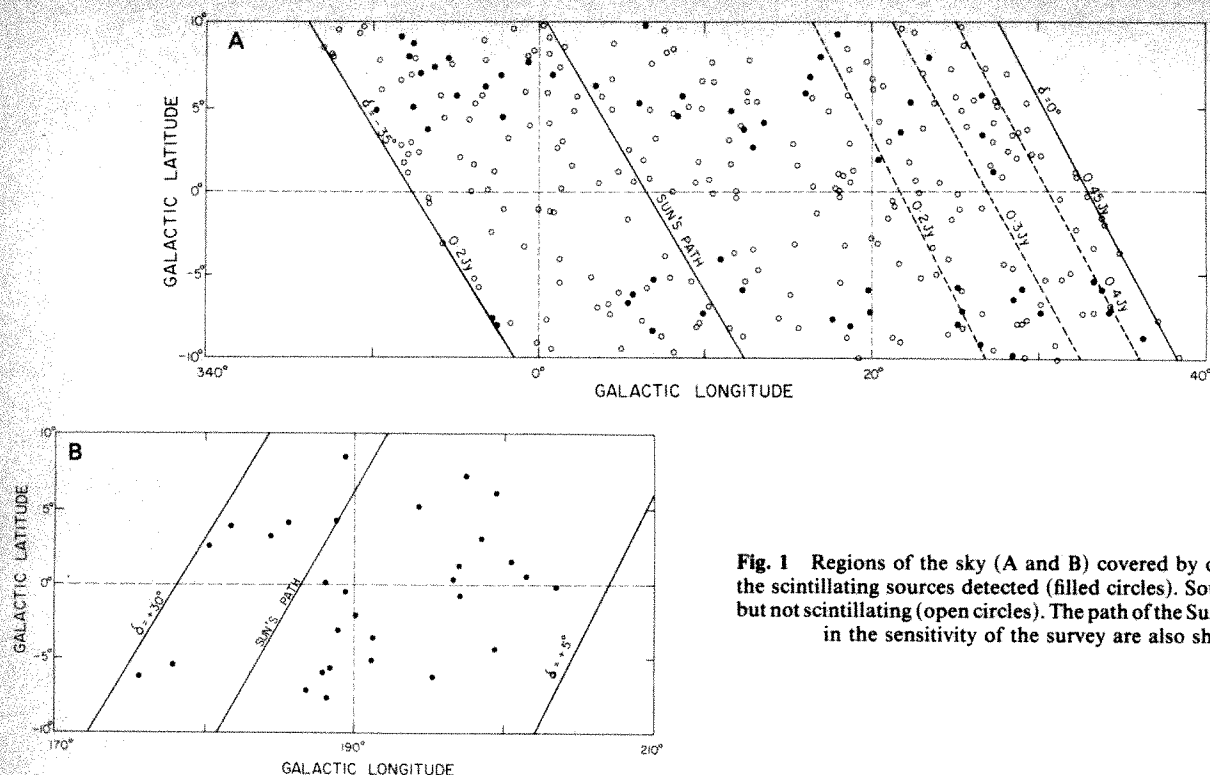


Fig. 1 Regions of the sky (A and B) covered by our survey and the scintillating sources detected (filled circles). Sources observed but not scintillating (open circles). The path of the Sun and variation in the sensitivity of the survey are also shown.

greater than 10° , ΔS of a point source decreases as the angular distance between the Sun and the source increases. As the Sun never comes close to sources at high ecliptic latitudes, the sensitivity of the IPS technique decreases with ecliptic latitude. The maximum absolute value of the ecliptic latitudes of the surveyed regions is about 25° , so that over the surveyed regions the sensitivity to point sources falls at most by 2.5 (ref. 6). Further, because of diameter blurring¹, the sensitivity of the IPS technique decreases as the angular size of the source increases and is too low, at 327 MHz, to be useful for sources with diameter greater than about 0.5 arc s unless the source has a large flux density.

Results

The boundaries of regions A and B and the detected positions of the scintillating sources are shown in Fig. 1. There is a striking difference in the distribution of scintillating sources in the two regions. In the galactic anticentre direction we detected scintillating sources even at $b = 0^\circ$, but in the galactic centre region we did not detect any scintillating source of $|b| < 1.5^\circ$. We divided the latitude range of the regions surveyed into three equal zones, each of width 3.3° (Table 1). Although the number of catalogued sources in the lowest latitude zone in region A was about 60% smaller than in the higher latitude zones because of effects of confusion in the Clark and Crawford⁴ survey, the number of scintillating sources in this zone is six times smaller. In contrast, in the anticentre direction, we detected three times more scintillating sources in the lowest latitude zone even though the survey

was over a smaller area of the sky and is not complete. As the sensitivity of our survey does not depend on galactic latitude, the observed deficiency of sources at low latitudes in region A is a real effect which must be explained.

We next plotted the dependence of the diameter of the scintillating component on galactic latitude for four galactic longitude ranges (Fig. 2). As the number of sources at low latitudes is small, we included four sources which were scintillating, but did not satisfy the criteria set for inclusion in our survey. Two of the sources (1740-261 and 1855-016) have scintillating flux marginally less than the survey limit of 0.2 Jy, whereas the other two (1903+015 and 1915+062) were north of the northern boundary of our survey. In the longitude range $30^\circ > l > -10^\circ$ where the deficiency of scintillating sources for $|b| < 3.3^\circ$ is most pronounced, all six detected scintillating sources in this latitude range have angular diameters larger than 0.4 arc s. As the sensitivity of the IPS technique for sources in this diameter range is less than that for point sources, this argues strongly against the observed deficit of sources at $|b| < 3^\circ$ being an artefact caused by the lower sensitivity of our survey in the galactic plane. The observed large values of the angular sizes of the scintillating sources at low latitudes (Fig. 2a, b) are probably the effects of ISS broadening in these longitude ranges, the deficiency of scintillating sources being a consequence of this broadening. The absence of scintillating sources with $|b| < 1.5^\circ$ requires that the ISS angle at these latitudes is > 1 arc s, consistent with previous observations⁸, at 408 MHz and low galactic latitudes, of enhanced scattering along lines of sight passing through the inner Galaxy.

In the presence of ISS broadening, the observed brightness distribution of a source is the convolution of the true distribution with the point spread function due to the ISM. From our observations alone, we are unable to determine the ISS angle for each source as we do not know the intrinsic sizes of the detected scintillating sources. In principle, one could determine the ISS angle if one had made IPS observations at two frequencies, as the scattering angle increases roughly as the square of the wavelength whereas the intrinsic size of the sources changes more slowly. However, as the intrinsic sizes of the scintillating sources at 327 MHz are likely to be between 0 and 1 arc s (Fig. 2d), the lower envelope of the points in Fig. 2a and b is a statistical measure of the ISS angle in these longitude ranges.

Table 1 Break-up of the number of sources at different galactic latitudes

Latitude range	Number of compact sources detected		Number of catalogued sources studied	
	Galactic centre region A	Anticentre region B*	Region A	Region B
$0^\circ < b \leq 3.3^\circ$	5	17	97	ND
$3.3^\circ < b \leq 6.7^\circ$	31	11	146	ND
$6.7^\circ < b \leq 10^\circ$	30	7	141	ND

* Surveyed areas at different latitudes not equal. ND, not done.

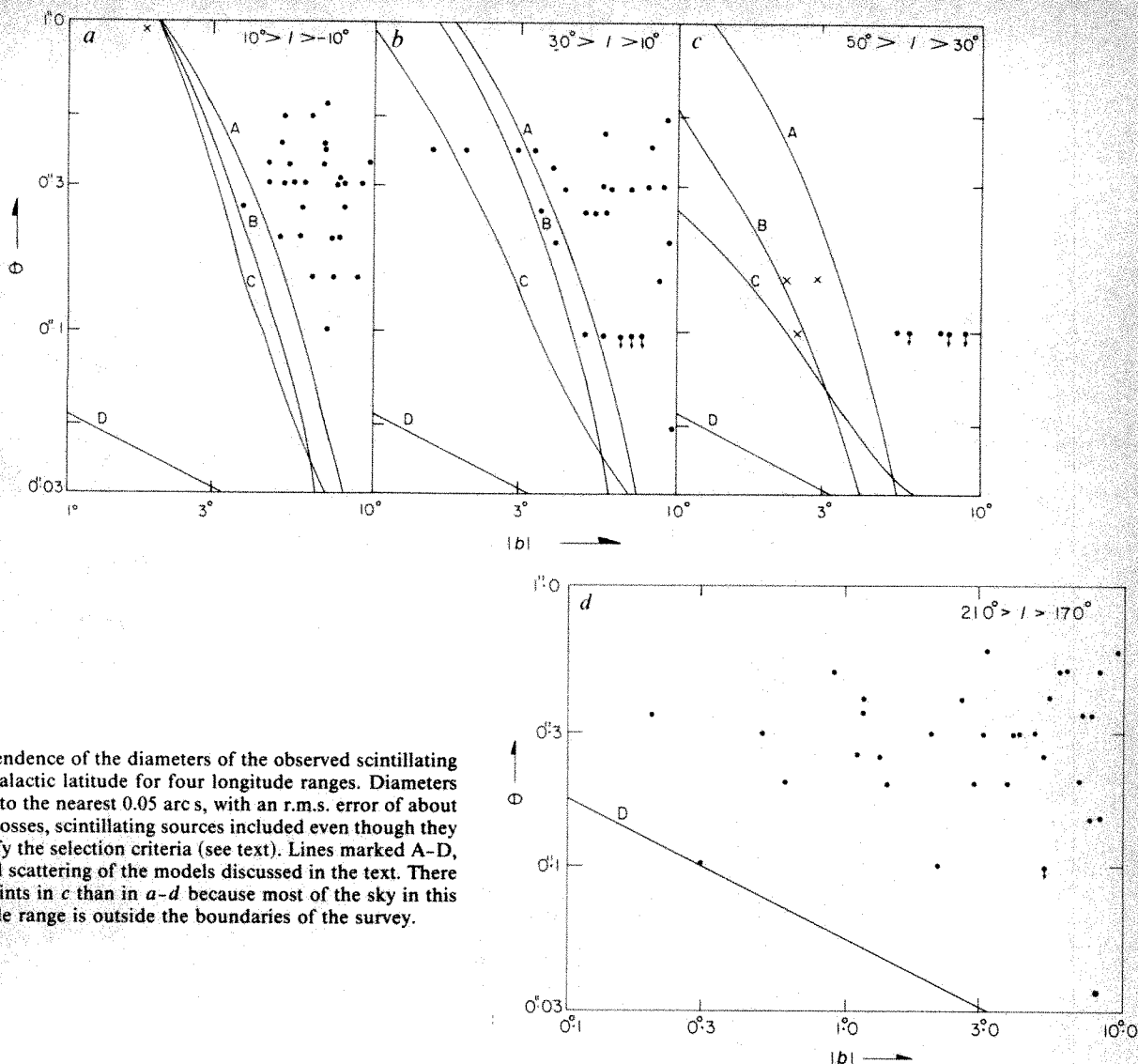


Fig. 2 Dependence of the diameters of the observed scintillating sources on galactic latitude for four longitude ranges. Diameters are rounded to the nearest 0.05 arc s, with an r.m.s. error of about 0.05 arc s. Crosses, scintillating sources included even though they did not satisfy the selection criteria (see text). Lines marked A-D, the predicted scattering of the models discussed in the text. There are fewer points in *c* than in *a-d* because most of the sky in this longitude range is outside the boundaries of the survey.

Where scintillating sources are systematically absent, the apparent angular sizes of sources are larger than 1 arc s. The lower envelope of the points in Fig. 2*a* and *b* increases as $|b|$ decreases. The magnitude of ISS broadening varies with both galactic latitude and longitude and its variation with galactic latitude is faster than $(\sin |b|)^{-1/2}$ (line D in Fig. 2). This rules out models of the ISM in which the scattering strength is independent of the galactic radius and the distribution perpendicular to the galactic plane is either uniform or exponential.

Two-component model for scattering

From an analysis of the 80 MHz Cambridge IPS survey supplemented with observations at 151 MHz, it has been concluded that scattering material in the ISM has scale height perpendicular to the galactic plane between 400 and 700 pc (ref. 9). At 80 MHz, for $|b| > 10^\circ$, the ISS angle is given by

$$\theta_{\text{scat}}(\text{arc s}) = 0.12(\sin |b|)^{-1/2} \text{ for } |b| > 10^\circ$$

(ref. 10), after correction for IPS diameters described in ref. 11. As this result is valid only for high galactic latitudes, it is sensitive to the scattering properties of the interstellar medium within about 2 kpc from the Sun. The properties of the local interstellar medium derived from this survey are in general agreement with those estimated from the dispersion and scattering properties of nearby pulsars. The variations of the ISS angle with wavelength depends on the shape of the spectrum of the irregularities responsible for the scattering¹². For a gaussian spectrum the ISS angle increases as $(\text{wavelength})^2$ whereas for a Kolmogorov spectrum it is related to $(\text{wavelength})^{2.2}$. The

extrapolation of the 80 MHz result to $|b| < 10^\circ$ after scaling to 327 MHz as $(\text{wavelength})^2$ giving the largest value for the scattering angle is shown in Fig. 2, line D. At very low galactic latitudes, the ISS angle could be less than this line because of the finite radial extent of our Galaxy. Over most of the sky, the predicted scattering angle is less than or comparable with the resolution limit of the IPS technique at 327 MHz (0.05 arc s), and so our survey should not see this scattering. This is consistent with our observations in the galactic anticentre direction, where we see no deficiency of scintillation sources at low galactic latitudes; within the uncertainty of the measurements there is no obvious lower envelope to the observed angular diameter significantly larger than the resolution limit of the IPS technique. However, in the galactic centre directions at low latitudes, the observed scattering is much larger than predicted. The fact that effects of ISS are seen in our survey at 327 MHz shows that this model for scattering in our Galaxy is inadequate at low latitudes. To explain our observations we need an additional component of the ISM whose scattering strength is both much larger and also confined to the inner parts of the Galaxy.

If the distribution of the strength of scattering in our Galaxy were known, the expected angular broadening for any given line of sight could be calculated easily. However, the inverse problem of determining the distribution of the scattering strength from the measured angular broadening in different directions is not easily solved, especially in our case where the angular broadening is measured only in a few directions. To understand the properties of the scattering region, we have compared our observations with the angular broadening predicted by some

simple models. The limitation of such an approach is that we cannot be sure of the uniqueness of the derived model.

For our calculations we have made the following assumptions: (1) The wave number spectrum of the plasma density fluctuations responsible for the scattering is a power law of the form C_N^2 (wavenumber) $^{-11/3}$ so that, following ref. 12, the half-power scattering diameter is given by

$$\theta_{\text{scat}}(\text{arc s}) = 1.72 \times 10^{-2} (\lambda_m)^{-2.2} \left[\int_0^s C_N^2(s') ds' \right]^{0.6}$$

where C_N^2 is related to the magnitude of the density fluctuations, λ_m the wavelength of observation in metres and s' the distance along the line of sight, measured in parsecs.

(2) We ignore any possible clumping of the scattering material and assume it is homogeneously distributed in the ISM.

(3) The Galaxy has cylindrical symmetry about the galactic centre and reflection symmetry about the galactic plane. Thus C_N^2 is only a function of r , the distance from the galactic centre, and $|z|$, the height above the galactic plane. We assume that C_N^2 varies exponentially with z with a scale height z_0 that is independent of r . Thus C_N^2 can be expressed as

$$C_N^2(r, z) = C_N^2(r) \exp(-|z|/z_0)$$

For $C_N^2(r)$ we have assumed three simple models (Table 2).

(4) The Sun is located at $z=0$ and $r=10$ kpc.

(5) All the scintillating sources we have seen are extragalactic.

For each of these models, and for a range of r_0 and z_0 , we computed the scattering angle for lines of sight in different directions. We fixed C_0^2 by requiring that all models give 1 arc s scattering at $l=2^\circ$, $b=2^\circ$, close to the observed parameters of 1740–261, the scintillating source whose line of sight is most close to the galactic centre. The effect of changing the normalization is to slide the model curves vertically in the plots. The goodness of fit was judged by comparing the predicted scattering angles with the lower envelope of the points in Fig. 2. Models with $z_0 \sim 500$ pc are unable to reproduce the observed variation of scattering angle with galactic latitude and longitude. To explain the sharp decrease of the ISS angle from 1 arc s at $|b| \sim 2^\circ$ to ~ 0.1 arc s at $|b| \sim 5^\circ$ in Fig. 2a, these models require the region of enhanced scattering to be confined to within 3 kpc of the galactic centre; thus, they cannot explain the enhanced scattering seen at longitudes as large as 35° . To explain both these observations, we must decrease z_0 . Because of the uncertainty in the scattering angle for $|b| < 1.5^\circ$, where no scintillating sources were seen, we could not uniquely determine z_0 and r_0 , even for the simple models we have considered. However, as most of the acceptable models had z_0 of 50–100 pc, we have assumed $z_0 = 75$ pc and determined r_0 (Table 2). The predicted variation of the scattering angle with latitude is shown in Fig. 2a–c, lines A–C. (The scattering predicted by the models is outside the range of Fig. 2d, hence there are no lines here.)

The constant scattering model is unable to reproduce the observed decrease in the scattering angle with galactic longitude. From our data alone we are unable to distinguish between the linearly-decreasing and the exponential models. The exponential model produces more scattering along the line to the galactic centre, but even this is less than half the observed scattering angle scaled to 327 MHz (ref. 13). If, ignoring the radial decrease in the scattering strength, we assume that material up to two

scale heights in z (13.5%) contributes to the observed scattering, the absence of any estimate of the scattering angle for $|b| < 2^\circ$ implies that our observations are sensitive only to material within 4 kpc of the Sun. Thus, our data shows that the strength of scattering must decrease with r for $r > 6$ kpc. Measurements of the scattering angle close to $|b| = 0^\circ$ would elucidate the scattering strength closer to the galactic centre.

Discussion

The scattering strength of the second component of the interstellar medium at low galactic latitudes is much larger than previously suggested¹⁰ ($C_N^2 \sim 10^{-4} \text{ m}^{-6.67}$; ref. 12). Pulse broadening and interstellar scintillation observations of pulsars whose distance is greater than 2 kpc show that C_N^2 increases with distance^{12,14}, interpreted as owing to enhanced scattering in a component with $z_0 \sim 100$ pc^{14,15}. Although the earlier model¹⁰ predicts extremely small values for the ISS angle at cm wavelength, high frequency VLBI observations^{16,17} have shown much larger scattering angles for some sources at low latitudes. As there are only very few such observations, however, they have been explained by the sources lying in directions where the line of sight passes through dense regions of the interstellar medium, such as HII regions. VLBI observations of the galactic centre¹³ show a component whose diameter increases roughly as the square of the wavelength, as expected for ISS. However, the scattering angle is four orders of magnitude larger than predicted previously¹⁰, leading to the suggestion that most of the observed scattering takes place within a few parsecs of the galactic centre itself¹⁸ and not in the general interstellar medium. The angular sizes of hydroxyl radical maser sources is larger for more distant sources¹⁹, suggesting that these sources are affected by general interstellar medium scattering, but that the magnitude of this scattering is much larger than predicted¹⁰. VLBI observations at 408 MHz of a sample of 29 low-latitude compact sources⁸ shows that the ISS angle for lines of sight in the inner Galaxy is greater than 0.3 arc s, whereas in the anti-centre direction it is hardly detectable. In all the above examples, the enhanced scattering is seen when the line of sight passes through the inner Galaxy close to the galactic plane, and thus can be explained by scattering in a component of the type required. Although our estimates of the scattering properties of this component may change with more and better observations, it is clear that any general model for scattering in our Galaxy must have at least two components.

As the plasma density fluctuations responsible for scattering are probably proportional to mean density, the mean plasma density is considerably higher also in the region of enhanced scattering. Analysis of the dispersion measures of pulsars shows that the mean density is larger in the inner parts of our Galaxy^{20,21}. As most of the high dispersion pulsars ($DM > 200 \text{ pc cm}^{-3}$) are seen through the inner Galaxy, the distance derived from the dispersion measures is strongly dependent on reliable estimates of the plasma density in this region. Pulse broadening caused by enhanced scattering in the inner parts of the Galaxy leads to an observational selection effect against short period pulsars which could be important in existing pulsar surveys and affect our understanding of the distribution of pulsars in our Galaxy. Searches for millisecond pulsars in the inner parts of our Galaxy could be limited by this pulse broadening even at high frequencies.

As the presence of HII regions along the line of sight increases considerably the observed scattering²², it has been suggested that the enhanced scattering originates entirely from such regions^{8,14}. We have assumed that the scattering material is uniformly spread through the interstellar medium; by estimating the actual scattering angle for all the detected compact sources and by studying the variation of ISS angle with position in the sky, it will be possible to test this hypothesis. The observed distribution of the HII region²³ and radio recombination lines²⁴ suggest the existence of dense thermal plasma in the inner parts of the Galaxy with z_0 of 50–100 pc. It is interesting to speculate whether the region of enhanced scattering is associated with

Table 2 Models for $C_N^2(r)$ and estimated parameters for $z_0 = 75$ pc

Model	Functional form	$C_0^2(\text{m}^{-6.67})$	$r_0(\text{kpc})$
Constant	$C_N^2 = C_0^2$ if $r < r_0$ $= 0$ if $r > r_0$	2.4	7
Linearly decreasing	$C_N^2(r) = C_0^2(1 - r/r_0)$ if $r < r_0$ $= 0$ if $r > r_0$	8.3	7
Exponential	$C_N^2(r) = C_0^2 \exp(-r/r_0)$	56.7	1

this dense plasma. Although in our models we have assumed that scattering strength decreases monotonically with distance from the galactic centre, the estimated density of the thermal plasma shows a peak 4–6 kpc from the galactic centre. The predicted variation of the ISS angle with longitude for $|b| < 0.5^\circ$ is different for these two cases, we cannot distinguish between

them, as estimates of angular broadening occur only to $|b| \sim 2^\circ$. Extension of our observations down to $b = 0^\circ$, either by making IPS observations at high frequencies or by other techniques, will improve considerably our model for C_N^2 . This may allow us to understand the relation between the density fluctuations and the physical processes in the interstellar medium.

Received 20 June; accepted 18 September 1984.

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Platelet-derived growth factor induces rapid but transient expression of the *c-fos* gene and protein

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Exposure of quiescent mouse fibroblasts to platelet-derived growth factor induces mRNA from the c-fos proto-oncogene within 10 min followed by synthesis of nuclear c-fos proteins. These are amongst the earliest described nuclear events that follow a mitogenic stimulus.

DESPITE an extensive knowledge of the molecular architecture of the proto-oncogenes (*c-onc*), little is known about the functions of their products^{1–3}. The *c-sis* gene codes for a subunit of platelet-derived growth factor (PDGF)^{4,5} and the *c-erb-B* gene may encode the epidermal growth factor (EGF) receptor⁶, suggesting roles for these proto-oncogenes in the regulation of cell growth. We are studying the proto-oncogene *c-fos*, whose viral cognate, *v-fos*, is the transforming gene of two murine retroviruses that cause osteosarcomas with short latency and transform fibroblasts in culture^{7–12}. The *c-fos* gene is conserved among vertebrates^{9,13} and differentially expressed during development¹⁴, suggesting that its product is required for growth or differentiation of certain tissues. The *c-fos*-specific RNA is not expressed in most pre- or postnatal mouse tissues. Expression of a 2.2-kilobase (kb) *c-fos* RNA has been detected, however, in extra-embryonic tissues, adult bone marrow and monocytes^{3,14–17}.

The predicted product of the *c-fos* gene differs from the *v-fos* gene product at its carboxy terminus¹⁰. These differences do not seem to be important for transformation as the *c-fos* gene can transform fibroblasts if first modified in two ways: (1) by the addition of a transcriptional enhancer element and (2) by the replacement of 3'-noncoding sequences with heterologous viral sequences¹⁸. The latter modification is thought to interrupt an interaction between sequences in the 3'-coding and noncoding sequences that may inhibit translation¹⁸. Neither change affects the coding region. Thus, rat 208F fibroblasts can be transformed by a hybrid gene containing *c-fos* promoter and coding sequences linked to viral 3'-untranslated sequences containing an enhancer¹⁸. These cells (R-MMV cells) contain a series of proteins differing in post-translational modification, that are totally encoded by the introduced mouse *c-fos* sequences¹⁹. Both *c-fos* and *v-fos* proteins are nuclear¹⁹.

Another proto-oncogene, *c-myc* (whose product is also a nuclear protein^{20,21}), is expressed transiently when fibroblast cultures are exposed to certain polypeptide mitogens²². We have

therefore examined the expression of the resident *c-fos* gene in normal mouse fibroblasts and find that even though *c-fos* mRNA is barely detectable in quiescent, confluent cultures of NIH 3T3 or BALB/c/3T3 cells, it is rapidly induced following exposure to any of the mitogens PDGF, fibroblast growth factor (FGF) or tetradecanoylphorbol-13-acetate (TPA). Expression of *c-fos* mRNA is maximal at 20 min and decreases thereafter. Synthesis and post-translational modification of *c-fos* proteins are readily detectable within the first 30 min of mitogen stimulation, are maximal by 60 min and then decrease. The rapidity of the response and our detection of *c-fos* mRNA induction even when protein synthesis is inhibited, suggest that this is a primary event in the pleiotropic response to mitogens.

Rapid induction of *c-fos* mRNA

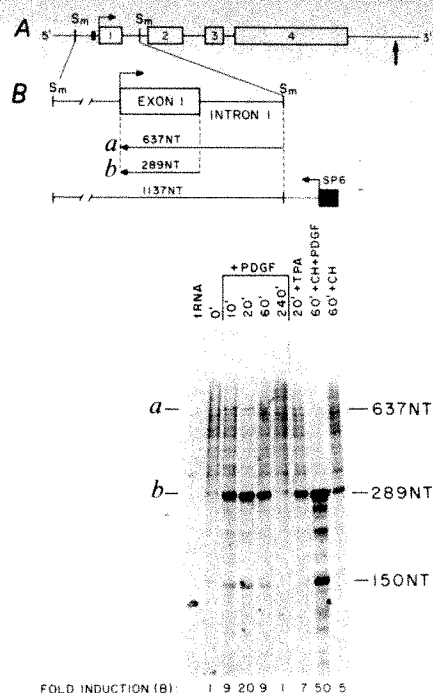
We exposed confluent cultures of NIH 3T3 cells to 0.83 nM PDGF for 4 h in the continuous presence of 35 μ M cycloheximide, which inhibited protein synthesis by >95%. PDGF alone increased the content of *c-fos* RNA threefold, the combination of PDGF and cycloheximide increased the *c-fos* RNA level 10-fold and cycloheximide alone increased the level twofold. Puromycin also augmented the increase in *c-fos* RNA. This suggested that protein synthesis was unnecessary for, and might even inhibit, an increase in *c-fos* RNA content, for example, by attenuation of transcription and/or by stimulating *c-fos* RNA degradation.

Studies on differentiation of monocytic and myelomonocytic cells in response to TPA revealed that, in this system, *c-fos* gene transcription can be activated very rapidly²³. Accordingly, we tested whether brief exposure of resting fibroblast cultures to PDGF would induce *c-fos* RNA. The *c-fos* gene transcripts were characterized by hybridization with ³²P-labelled complementary RNA (cRNA) synthesized using SP6 phage polymerase²⁴, followed by ribonuclease digestion and electrophoresis of the protected cRNA. The cRNA probe was transcribed from a *Sma*I fragment of 1,137 nucleotides encompassing the putative murine

Fig. 1 Analysis of *c-fos* RNA. **A**, Molecular structure of the mouse *c-fos* gene based on nucleotide sequence analysis¹⁰. Expected sizes of the protected *c-fos* mRNA transcripts are indicated, as are the positions of the putative 5' cap ($\cap$), polyadenylation signal ($\uparrow$), TATA box ($\blacksquare$), exon ($\square$), intron ($-$), SP6 phage promoter ($\blacksquare$) and vector ($\cdots$). **B**, Analysis of *c-fos* transcripts. Total RNA from PDGF-treated cells was used for RNA protection experiments. Times of induction (min) and type of treatment are indicated. As a control for self-hybridization of the probe, one hybridization contained 10 μ g tRNA instead of cellular RNA. Fold induction is indicated at the bottom of the figure. *a*, Expected size 637 nucleotides; *b*, expected size 289 nucleotides. The sizes of the protected fragments were determined relative to denatured ³²P-end-labelled pBR322 *Taq*I fragments as size markers.

Methods: 150-mm dish cultures of NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS). After reaching confluence the medium was replaced with 20 ml of DMEM 0.5% CS for 1 day. Partially purified PDGF (0.4%, from CM-Sephadex³⁴; at this step of the purification, PDGF concentration assayed by mitogenesis equals the concentration estimated by radioreceptor assay³⁰) was dissolved in 1 mM acetic acid and added to a final concentration of 0.83 nM. The same volume of a solution of bovine serum albumin (BSA, 1 mg ml⁻¹) dissolved in 1 mM acetic acid was added to a control dish. Cycloheximide (35 μ M) was added 2 min before PDGF. TPA (250 μ g ml⁻¹) was added from a stock solution in dimethyl sulphoxide (DMSO); the final DMSO concentration was 0.25%. After induction the cells were lysed in 4 M guanidine thiocyanate and total RNA was isolated⁴⁶. RNA was quantitated by measuring A₂₆₀. A 1,137-bp *Sma*I-*Sma*I fragment, spanning the 5'-untranscribed region and the first exon and part of the first intron of the mouse *c-fos* gene, was cloned in the *Sma*I site of the expression vector pRVII7Z2 (provided by Dr Angerer). To generate run-off transcripts, the plasmid was linearized by *Hind*III, which cuts just downstream of the inserted *c-fos* sequence relative to the site of the SP6 phage promoter in pRVII7Z2. cRNA run-off transcripts were synthesized in a reaction mixture containing 40 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 10 mM dithiothreitol (DTT), 50 U ml⁻¹ RNasin (Promega Biotec), 400 μ M GTP, ATP and CTP, 13 μ M α -³²P-UTP (650 Ci mmol⁻¹; ICN), 1 μ g linearized template and 1,000 U ml⁻¹ SP6 phage polymerase (isolated according to Butler and Chamberlain⁴⁷) for 1 h at 40 °C. Total RNA (10 μ g) was mixed with 5 ng ³²P-labelled cRNA (specific activity 109 d.p.m. μ g⁻¹) in 50 μ l 0.4 M NaCl, 1 mM EDTA, 80% formamide, 40 mM PIPES, pH 6.5. The mixture was heated at 85 °C for 5 min and incubated for 4 h at 37 °C, then for 10 h at 30 °C. After hybridization, 300 μ l of RNase digestion buffer (5 mM EDTA, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5) were added containing 30 μ g ml⁻¹ RNase A and 2 μ g ml⁻¹ RNase T1 and incubated for 30 min at 30 °C. RNase digestion was stopped by adding SDS (final concentration 0.7%) and proteinase K (0.15 μ g ml⁻¹) and incubation was continued for 30 min at 37 °C. The samples were extracted once with phenol/chloroform and ethanol-precipitated with 10 μ g tRNA as carrier and 1.0 M NH₄OOCCH₃. The precipitates were dissolved and re-precipitated with 0.5 M NH₄OOCCH₃. Dried pellets were resuspended in 5 μ l formamide/dye mixture and loaded on a 5% sequencing gel containing 8.3 M urea and 90 mM Tris-borate pH 8.3; 2.5 mM EDTA. The gel was exposed with a fluorescent screen for 3.5 h at -70 °C.

c-fos gene promoter, the first exon and part of the first intron¹⁰ (Fig. 1A). If transcription starts at the presumed 5'-cap site then the primary, unspliced transcripts should protect 637 nucleotides of the probe and the spliced mature mRNA should protect 289 nucleotides of the cRNA from ribonuclease digestion (Fig. 1A). Ribonuclease-resistant hybrids of ~289 nucleotides were formed with RNA extracted from PDGF-treated NIH 3T3 cell cultures (Fig. 1B); this band was undetectable with RNA from untreated cultures. Quantification of this band indicated at least 20-fold induction of *c-fos* mRNA by 20 min exposure of cells to PDGF. Addition of PDGF plus cycloheximide for 60 min resulted in a 50-fold increase in spliced *c-fos* RNA relative to a ninefold increase with PDGF alone. By measuring the radioactivity in the 289-nucleotide fragment, we estimate that after a 20-min exposure to PDGF, 0.0001% of NIH 3T3 cell RNA (0.005% mRNA) is *c-fos* mRNA. Assuming a cellular RNA content of 6 pg, this corresponds to about 5–10 copies of *c-fos* mRNA per



cell after 20 min of induction. Essentially identical kinetics of induction were observed with RNA from stimulated cultures of BALB/c/3T3 cells.

Protection of two other fragments was observed in the cRNA protection experiment (Fig. 1B). One fragment, corresponding in size to that expected for unspliced *c-fos* RNA (*a*), was not detected with RNA from either unstimulated cells or cells treated with PDGF for 4 h. With all other samples the amounts of the ~637-nucleotide fragment were similar. Cycloheximide did not increase the amount of this fragment, suggesting that transcription rate was unaffected. Protection of a cRNA fragment of ~150 nucleotides was also prominent with samples in which the 289-nucleotide fragment was detected (Fig. 1B). The identity of the smaller fragment is unknown but it could represent transcription from alternate promoter sites or splicing within the usual first exon. Alternatively, it could be an artefact of the analysis, as several other bands of varying intensity were also observed.

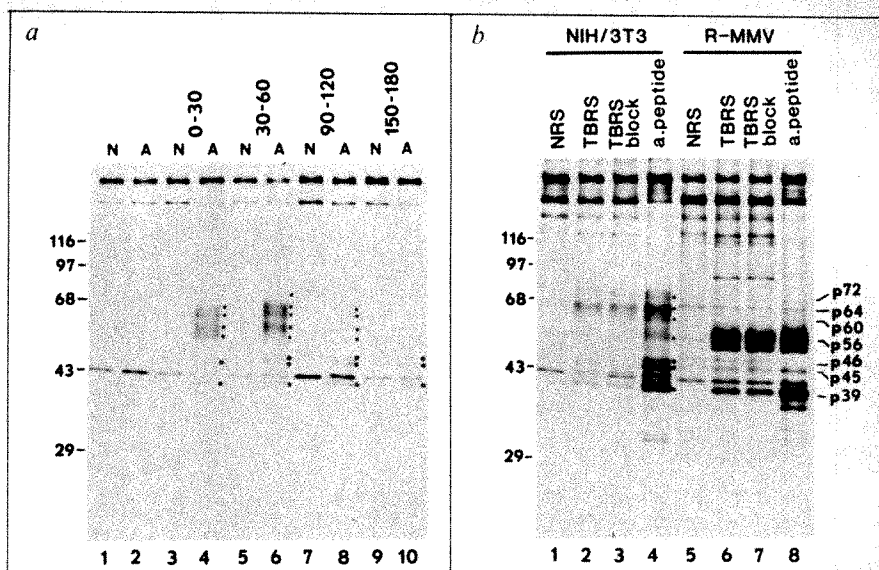
Expression of *c-fos* proteins

We next looked for protein products of the *c-fos* gene by immunoprecipitation of ³⁵S-methionine-labelled cultures using an antiserum directed against a 26-amino acid peptide (M2 peptide, residues 127–152 of the 380-amino acid predicted *c-fos* protein)^{10,25}. BALB/c/3T3 cell cultures were labelled with ³⁵S-methionine for 30 min, starting at various times after addition of 0.67 nM PDGF. Six to eight polypeptides were immunoprecipitable from PDGF-treated but not from control cultures (Fig. 2a). Some of these proteins are *c-fos* gene products and some are unrelated (Fig. 3). Exposure to PDGF for as little as 30 min induced labelling of these immunoprecipitated proteins with hardly any change in the overall rate of protein synthesis. Labelling was maximal by 60 min after addition of PDGF and was decreased markedly 3 h after PDGF addition. The relative incorporation of ³⁵S-methionine into each protein varied with time; the apparent sizes of all the species of relative molecular mass (*M_r*) between 56,000 (56K) and 72K seemed to increase slightly 30–60 min after PDGF addition. None of these proteins was immunoprecipitated using non-immune rabbit serum (Fig. 2a), nor if excess M2 peptide was used to block specific binding to M2-peptide antiserum (Fig. 4a).

Previous analysis of R-MMV cells has shown that *c-fos* proteins are very heterogeneous due to post-translational modifica-

Fig. 2 Synthesis of *c-fos* proteins in PDGF-treated 3T3 cells. **a**, Time course of *c-fos* protein synthesis; **b**, specificity of immunoprecipitation.

Methods: **a**, BALB/c/3T3 cell cultures were treated with 0.67 nM pure PDGF for 0 (lanes 3, 4), 30 (lanes 5, 6), 90 (lanes 7, 8) or 150 min (lanes 9, 10) before the addition of 100 μ Ci of ^{35} S-methionine. Another culture received an equivalent volume of BSA in 1 mM acetic acid for 30 min before labelling (lanes 1, 2). After a further 30 min of incubation, cultures were washed, lysed and one-third volumes were immunoprecipitated with 1 μ g IgG equivalent of non-immune rabbit serum (N, lanes 1, 3, 5, 7 and 9) or 1 μ g affinity-purified IgG to M2 peptide (A, lanes 2, 4, 6, 8 and 10). Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis. Proteins related to *c-fos* are marked by a closed square, unrelated proteins by an open circle. A band of M_r 43,000, observed in all samples, is probably actin. **b**, Portions of a NIH 3T3 cell lysate (labelled for 30 min starting 30 min after addition of 0.83 nM pure PDGF) and a R-MMV cell lysate (labelled for 30 min) representing 1/20 of a 35-mm dish culture in each case, were immunoprecipitated with 1 μ l normal rat serum (lanes 1, 5), 1 μ l TBRs (lanes 2, 6), 1 μ l TBRs mixed with 1 μ g M2 peptide (lanes 3, 7) or 0.3 μ g affinity-purified IgG to M2 peptide (lanes 4, 8). Proteins precipitated from PDGF-treated NIH 3T3 cells by antiserum to M2 peptide are indicated at the right and by a closed square (*c-fos*-related proteins) or open circle (proteins not related to *c-fos*) adjacent to lane 4. For labelling conditions see Fig. 3 legend. Except where noted, confluent 35-mm dish cultures of 3T3 cells were incubated in 1 ml DMEM containing 1% normal methionine concentration and 0.5% CS for 40–48 h. Additions of PDGF (essentially homogeneous, purified through phenyl-Sepharose³⁴, dissolved in 1 mM acetic acid containing 1 mg ml⁻¹ BSA), or an equal volume of 1 mg ml⁻¹ BSA in 1 mM acetic acid, and ^{35} S-methionine (100 μ Ci of $\sim$ 1,000 Ci mmol⁻¹; Amersham/Searle) were made directly to the medium. Cultures were lysed after washing with cold Tris-buffered saline by adding 0.5 ml of RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 100 U ml⁻¹ Trasylol, 10 mM sodium phosphate, pH 7.0) and scraping. Lysates were clarified at 20,000g for 60 min at 4°C. IgG or antisera were added as indicated. After 1 h at 0°C, 1 mg Pansorbin (Calbiochem) was added for 1 h. Immunoprecipitates were centrifuged through a solution of 10% sucrose in RIPA, then washed repeatedly by centrifugation in RIPA⁴⁸. Immunoprecipitations with rat antitumour serum or normal rat serum utilized goat antiserum to rat IgG added 30 min before Pansorbin. Immunoprecipitates were dissociated by incubation at 100°C for 2 min in 2% SDS, 20% 2-mercaptoethanol, 10% glycerol, 0.1 M Tris-HCl, pH 6.8, and one-half of each sample analysed on a SDS-polyacrylamide gel⁴⁹ (12.5% acrylamide/0.10% bis-acrylamide). Gels were stained to visualize marker β -galactosidase, phosphorylase, BSA, ovalbumin and carbonic anhydrase and impregnated with diphenyloxazole (PPO)⁵⁰. Dried gels were exposed to pre-sensitized film at -70°C. Exposure times: **a**, 10 days; **b**, 4 days.



tion, although there are several reasons to suspect that some of the apparently-induced proteins were not products of the *c-fos* gene¹⁹. First, antisera to M2 peptide, or sera from rats with *v-fos*-induced tumours (TBRs), can precipitate also an unrelated cellular protein, p39, from cells expressing either *c-fos* or *v-fos*^{19,25}. This protein appears to be associated with the *fos* gene products^{25,26}. Increased immunoprecipitation of labelled p39 might be expected following PDGF treatment, whether or not its synthesis is induced. Second, one mRNA induced after 3–4 h of exposure of BALB/c/3T3 cells to PDGF (cDNA clone pBC-JB)²⁷ shares a short region of homology with *c-fos*²⁸, hence this induced sequence has been named *r-fos*²⁸. Coincidentally, the M2 peptide encompasses the entire 12-amino acid region of *c-fos*/*r-fos* homology. Thus the antiserum to M2 peptide could potentially recognize a putative *r-fos* protein. (The complete *r-fos* coding sequence has not been determined and the size of its protein product is unknown.)

To elucidate the identities of the immunoprecipitated proteins, NIH 3T3 cells were labelled with ^{35} S-methionine for 30 min, starting 30 min after addition of 0.83 nM PDGF; lysates were immunoprecipitated with antiserum to M peptide (Fig. 2b, lane 4) or with TBRs (Fig. 2b, lane 2). TBRs specifically precipitated the 56–72K proteins recognized by M2-peptide antiserum but was less efficient at precipitating the presumed p39 proteins and failed to precipitate two proteins of M_r 45K and 46K (p45 and p46). These two proteins were not precipitated in appreciable amounts from R-MMV cells with either TBRs or M2-peptide antiserum (Fig. 2b, lanes 6 and 8). Some of the proteins precipitated with TBRs may be *r-fos* gene products, as in principle this antiserum could recognize the region of homology between *r-fos*, *c-fos* and *v-fos*. Therefore, we tested whether excess M2 peptide could block the precipitation by TBRs of any proteins,

and found that it could not (Fig. 2b, lanes 3 and 7), suggesting that none was recognized solely through the shared peptide region. Furthermore, tryptic peptide analysis confirmed that all four immunoprecipitated proteins of M_r 56–72K were homologous to each other and to two authentic murine *c-fos* gene products synthesized in R-MMV cells (Fig. 3e–h). Thus the different sizes of the *c-fos* products in NIH 3T3 cells and R-MMV cells presumably result from differential post-translational modification. The M_r 39K proteins precipitated from PDGF-stimulated NIH 3T3 mouse cells and from R-MMV rat cells were also highly conserved (Fig. 3a, b). p45 and p46 were very similar and also shared some peptides with p39 (Fig. 3c, d); their genetic origin is unknown.

PDGF and other mitogens

The dependence of p56–72 *c-fos* induction on PDGF concentration was investigated by labelling with ^{35} S-methionine for 30 min, starting 30 min after PDGF addition. With either NIH 3T3 cells (data not shown) or BALB/c/3T3 cells (Fig. 4a) *c-fos* protein synthesis was maximal at PDGF concentrations that saturate PDGF binding sites at 37°C (1.0 nM)^{29–31} and half-maximal at the concentration for half-maximal binding (0.3–0.5 nM)^{30,31}. These doses are greater than those required for stimulation of DNA synthesis (half-maximal below 0.1 nM; refs 32–34 and data not shown). p56–72 *c-fos* synthesis was detectable at 50% mitogenic doses of PDGF, however (Fig. 4a). The rate of p56–72 *c-fos* synthesis was difficult to quantify because of the number of species involved, but may approach 0.005% of total protein synthesis during the 30-min labelling period. Synthesis of p56–72 *c-fos* was also stimulated by basic pituitary FGF (120 ng ml⁻¹) or TPA (100 μ g ml⁻¹), but was increased only slightly by EGF (8.3 nM; Fig. 4b).

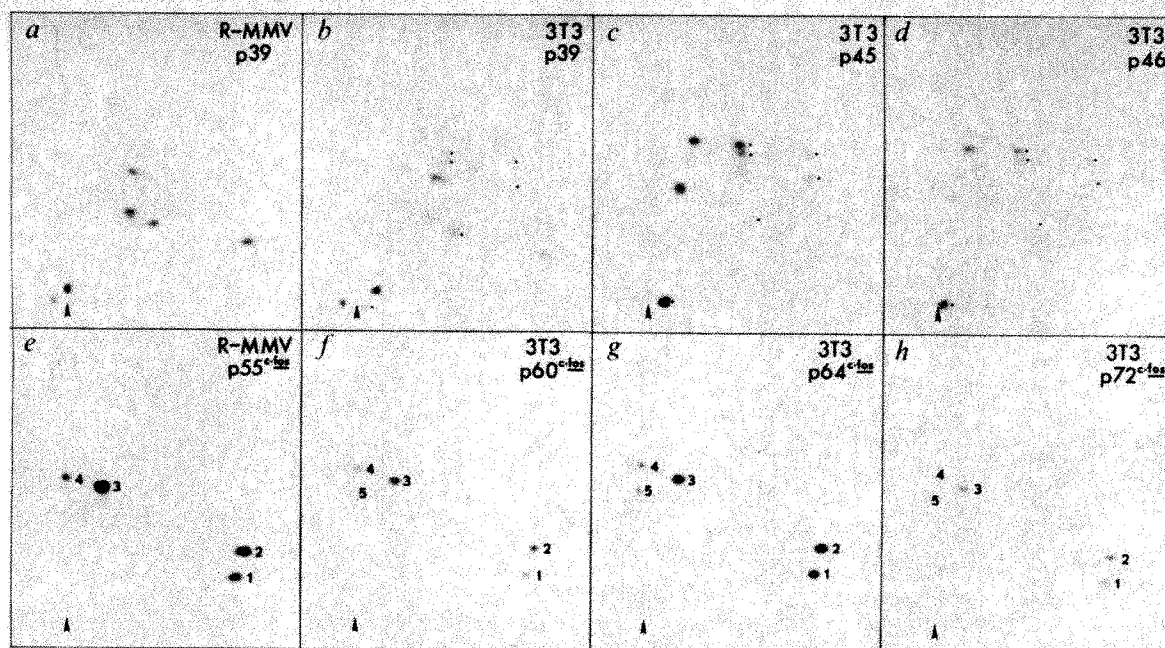
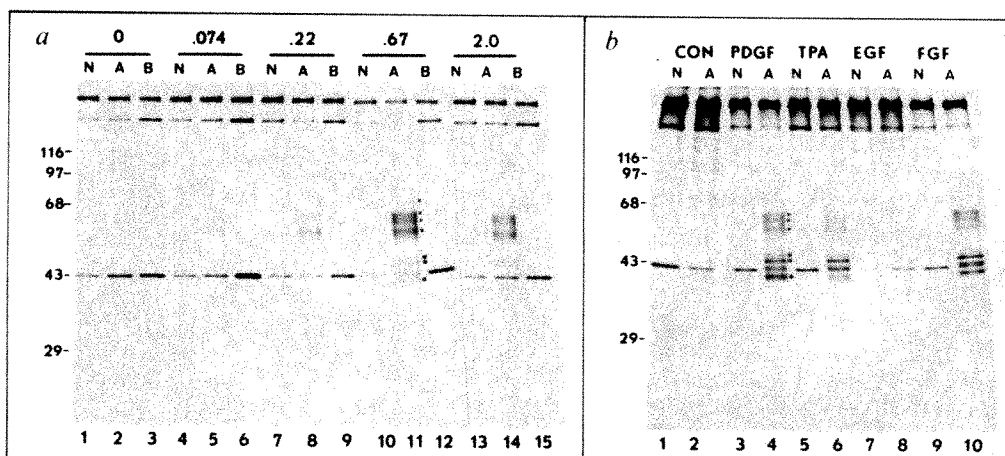


Fig. 3 Tryptic peptide maps of ^{35}S -methionine-labelled proteins immunoprecipitated from PDGF-treated cells. *a*, R-MMV cell p39 (1,720 c.p.m., 4-day exposure); *b*, NIH 3T3 cell p39 (1,100 c.p.m., 4 days); *c*, NIH 3T3 cell p45 (500 c.p.m., 8 days); *d*, NIH 3T3 cell p46 (600 c.p.m., 8 days); *e*, R-MMV cell p55^{c-fos} (960 c.p.m., 8 days); *f*, NIH 3T3 cell p60^{c-fos} (700 c.p.m., 8 days); *g*, NIH 3T3 cell p64^{c-fos} (860 c.p.m., 8 days); *h*, NIH 3T3 cell p72^{c-fos} (560 c.p.m., 8 days). Peptides in common between murine p39, p45 and p46 are indicated in *b*, *c* and *d* by closed circles (peptide maps of mixtures not shown). Peptides found in murine *c-fos* proteins are numbered 1-5 (*f*, *g*, *h*); all of these except number 5 co-migrate with peptides found in the murine *c-fos*-gene product synthesized in rat R-MMV cells (*e*) (peptide maps of mixtures not shown). From the murine *c-fos* gene sequence¹⁰, we predict that trypsin should generate three methionine-containing peptides small enough to be resolved in our analysis. One is found without mutation in *v-fos* (residues 149-153, MAAAK), one is slightly altered (1-16, single change, S to F) and one is significantly altered in charge (residues 109-113, AGMVK to AEMVK)¹⁰. Analysis of tryptic peptides from p55^{v-fos} showed the expected co-migrating peptide (number 1), peptides corresponding closely to peptides 3 and 4, and a new peptide more acidic than peptide 2 (data not shown). (Compare with ref. 51; peptide maps shown in inverse orientation.)

Methods: A confluent 35-mm dish culture of NIH 3T3 cells was incubated for 2 days in DMEM containing 1% normal methionine concentration and 0.5% CS. The volume was then reduced to 0.4 ml and 0.83 nM pure PDGF was added for 30 min, followed by 2 mCi ^{35}S -methionine for 30 min. A 35-mm culture of R-MMV cells (clone MMV6B) was incubated for 30 h in DMEM containing 1% normal methionine concentration and 5% dialysed CS before the volume was reduced to 0.4 ml and 1 mCi ^{35}S -methionine added for 30 min. Cultures were washed, lysed and four-fifths of each sample immunoprecipitated with 3 μg affinity-purified IgG to M2 peptide (see Fig. 2). Analytical immunoprecipitations of the remainder of each lysate are shown in Fig. 2*b*. The polypeptides indicated (Fig. 2*b*) were excised from the dried gel, eluted, oxidized with performic acid and digested with trypsin as described elsewhere⁵². Half of each digest was analysed by electrophoresis at pH 4.72 (anode at left, origin arrowed) and ascending TLC⁵³. Chromatograms were dipped in molten 2-methylnaphthalene containing 0.4% PPO and exposed to pre-sensitized film at -70°C .

Fig. 4 Induction of *c-fos* proteins by different concentrations of PDGF and other mitogens. *a*, Dose-dependence of *c-fos* protein synthesis; *b*, effects of other mitogens on *c-fos* protein synthesis.

Methods: *a*, BALB/c/3T3 cells were exposed to various concentrations of pure PDGF, diluted in 1 mM acetic acid containing 1 mg ml⁻¹ BSA, for 30 min before the addition of ^{35}S -methionine for 30 min. The final concentrations of PDGF were 0 (lanes 1-3), 0.074 nM (lanes 4-6), 0.22 nM (lanes 7-9), 0.67 nM (lanes 10-12) or 2.0 nM (lanes 13-15). Each lysate was immunoprecipitated with non-immune rabbit serum (N, lanes 1, 4, 7, 10 and 13), 1 μg affinity-purified IgG to M2 peptide (A, lanes 2, 5, 8, 11 and 14) or 1 μg IgG to M2 peptide preincubated with 3 μg M2 peptide (B, lanes 3, 6, 9, 12 and 15). All the samples were run on the same gel and exposed for 10 days, but lanes 10-12 and 13-15 have been rearranged in the figure. *b*, NIH 3T3 cells were exposed to BSA (lanes 1, 2), pure PDGF (2.5 nM, lanes 3, 4), TPA (0.1 mg ml⁻¹, lanes 5, 6), EGF (8.3 nM, lanes 7, 8) or FGF⁵⁴ (0.12 μg ml⁻¹, lanes 9, 10) for 30 min before the addition of ^{35}S -methionine for 30 min. Each sample was immunoprecipitated with non-immune rabbit serum (N, lanes 1, 3, 5, 7 and 9) or affinity-purified IgG to M2 peptide (A, lanes 2, 4, 6, 8 and 10). All samples were run on the same gel and exposed for 5 days.



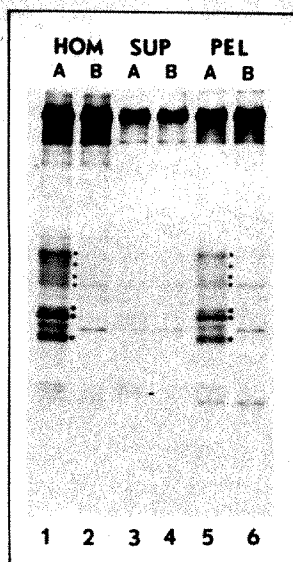


Fig. 5 Subcellular localization of induced *c-fos* proteins. A 35-mm dish of NIH 3T3 cells was treated with partially purified PDGF (0.83 nM) in 0.4 ml medium for 30 min before the addition of 300 μ Ci ^{35} S-methionine for 30 min. After washing with cold saline the cells were scraped from the dish in 0.4 ml of 1 mM DTT, 10 U ml $^{-1}$ Trasylol, 10 mM HEPES, pH 7.4 and homogenized in a Teflon/glass homogenizer. Half of the sample was removed and the remainder centrifuged at 600g for 5 min at 4°C. One volume of twice-concentrated RIPA buffer (see below) was added to each fraction. Following centrifugation, small aliquots of each fraction were analysed by SDS-polyacrylamide gel electrophoresis. Certain proteins were found in the supernatant but not in the pellet fraction, suggesting that contamination of the latter fraction with cytoplasm or unbroken cells was negligible (data not shown). Half of the remainder of each fraction—total homogenate (lanes 1, 2), supernatant (lanes 3, 4) and pellet (lanes 5, 6) was then immunoprecipitated with either 1 μ g affinity-purified IgG to M2 peptide (A, lanes 1, 3 and 5) or 1 μ g IgG to M peptide preincubated with 3 μ g M peptide (B, lanes 2, 4 and 6). The gel was exposed for 2 days.

Localization of induced *c-fos* proteins

Immunofluorescence and cell fractionation methods have shown that *c-fos* proteins synthesized in R-MMV cells are nuclear¹⁹. Fractionation of PDGF-treated NIH 3T3 cells by homogenization and low-speed centrifugation followed by immunoprecipitation, suggests that *c-fos* proteins in these cells are nuclear also (Fig. 5). All proteins specifically immunoprecipitated with antiserum to M2 peptide, including p56-72 *c-fos*, p39, p45 and p46 were found in the low-speed pellet fraction containing nuclei (Fig. 5). The induced *c-fos* proteins have not yet been detected by immunofluorescence.

Discussion

Within 10 min of treatment with 0.83 nM PDGF, a nine-fold induction of *c-fos*-specific mRNA is observed in NIH 3T3 cells (Fig. 1) and BALB/c/3T3 cells (data not shown). Exposure of BALB/c/3T3 cells to PDGF also induces *c-myc* RNA²². In our experiments, *c-myc* mRNA continues to accumulate for at least 4 h after PDGF stimulation of NIH 3T3 cells, whereas *c-fos* RNA is expressed only transiently. In other cell types, there is very rapid induction of the growth hormone gene by steroids and of the prolactin gene by EGF^{35,36}, but mRNA levels do not decrease for several hours.

In the presence of protein synthesis inhibitors, at least 50-fold induction of mature *c-fos* mRNA was detected in NIH 3T3 cells (Fig. 1) and BALB/c/3T3 cells (data not shown). Thus, like *c-myc*, *r-fos* and several other genes activated by PDGF, the *c-fos* gene is 'superinduced' when protein synthesis is blocked by cycloheximide^{22,28}. Cycloheximide may either cause stabilization of *c-fos* mRNA or enhance its *de novo* synthesis, for example through inhibition of the synthesis of a labile repressor

of *c-fos* gene transcription. Although the later possibility has been suggested to explain superinduction of *c-myc* gene²², our experiments support the former possibility for *c-fos*, as the amount of unspliced *c-fos* transcript was not increased in conditions of superinduction.

The synthesis of *c-fos* proteins is induced rapidly by PDGF (Fig. 2a), thus PDGF binding²⁹⁻³¹, signal relay to the nucleus, gene transcription, mRNA processing and transport and protein synthesis all must occur in 30 min. Clearly, the mechanism of signal transduction is very rapid, and may well be a direct response to PDGF binding. Indeed, both the concentration dependence and kinetics of *c-fos* gene activation correlate well with previous estimates of occupation of cell-surface PDGF receptors on NIH 3T3 cells at 37°C (ref. 31). The kinetics of the decline in *c-fos* mRNA synthesis coincide with internalization and degradation of bound PDGF^{29,30}. PDGF has many rapid effects on resting fibroblasts³⁷⁻³⁹. The transcription of *c-fos* must be induced by an effect of PDGF not detected with EGF. Potentially, the protein-tyrosine kinase activity of the PDGF receptor may have a different specificity from that of the EGF receptor, although the substrate specificity identified to date are common to both factors^{31,40}.

The *c-fos* and *v-fos* proteins are modified extensively after translation. In PDGF-treated NIH 3T3 or BALB/c/3T3 cells, at least four *c-fos*-encoded products could be detected (Figs 2, 3). There may be further modified *c-fos* proteins that are too heterogeneous to be detected or that may not be recognized by the peptide antiserum used in our studies. All four *c-fos* species (p56-72 *c-fos*) and the label in p39 disappeared equally rapidly with a half-life of ~2 h (data not shown). If, as we think, p39 is precipitated because of its association with *c-fos* proteins, then *c-fos* proteins are degraded rather than converted into more heavily modified forms. We estimate that the proteins accumulate to much less than 0.0005% of cell protein (~25,000 molecules per cell), but if only one of the many modified forms of p56-72 *c-fos* is active, then even smaller amounts of active gene product may be present. A small number of other mRNAs, including *c-myc* and *r-fos*, are induced by PDGF^{22,27,28}, although they may not be induced as rapidly as *c-fos*. One protein, CPI⁴¹, whose gene is unknown, is synthesized very rapidly after PDGF addition⁴¹, and is nuclear⁴², similar to products of *c-fos* and *c-myc*.

What is the biological significance of rapid *c-fos* RNA and protein synthesis in response to PDGF, FGF and TPA? Studies with BALB/c/3T3 cells have allowed the classification of mitogens as 'competence' or 'progression' factors⁴³ which cooperate for a full mitogenic response. The former category includes PDGF, FGF and TPA; the latter EGF and the insulin-like growth factors⁴³. This classification is not as clear in some other cell types, however, and it is interesting that we could observe induction of *c-fos* proteins with both NIH 3T3 and BALB/c/3T3 cells. The products of the *c-fos*, *c-myc* and other induced genes might all cooperate in some common cellular response or might each mediate a different biological response. Their nuclear localization suggests that they may affect nuclear functions, such as the activation of other genes. In the case of *c-myc*, fibroblasts constitutively synthesizing *c-myc* RNA have a decreased requirement for PDGF for growth⁴⁴. Perhaps constitutive synthesis of *c-fos* RNA and proteins in R-MMV cells also contributes a relaxation of competence factor requirements for cell growth.

Rapid induction of *c-fos* transcripts in PDGF-treated BALB/c/3T3 cells has been reported recently⁴⁵.

We thank Elaine Raines and Russell Ross for supplying both partially purified and pure PDGF, Denis Gospodarowicz for partially purified FGF, Chuck Van Beveren and Tom Curran for affinity-purified antiserum to M peptide, Liza Zokas and Tom Curran for TBRS, Dr R. Angerer for SP6 plasmids and Julie Baldwin for a clone of BALB/c/3T3 cells. We also thank Dick Mitchell for helpful discussions and Chuck Stiles for communication of unpublished results. This work was supported by grants from the NIH and ACS. W.K. was supported by a fellowship from KWF, the Dutch Queen Wilhelmina Fund.

Received 14 September; accepted 7 November 1984.

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Induction of c-fos gene and protein by growth factors precedes activation of c-myc

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Stimulation of fibroblasts with serum or purified growth factors leads to a dramatic induction of expression of both c-fos mRNA and protein within a few minutes, followed by activation of c-myc. This suggests that c-fos induction is a primary event and the earliest known effect on gene expression by growth factors.

RECENT evidence supports the hypothesis that cellular proto-oncogenes (c-onc genes) have key roles in the biochemical pathways controlling cell proliferation. Two distinct proto-oncogenes (c-sis and c-erb-B) control critical events in the transduction of extracellular growth signals into the cell. The c-sis gene probably encodes the platelet-derived growth factor (PDGF)^{1,2} and the c-erb-B gene product the epidermal growth factor (EGF) receptor³. For c-erb-B, the conversion of a cellular growth-factor receptor to an oncogene (v-erb-B) has been accomplished by loss of sequences in the regulatory ligand-binding domain³. This could lead to an unregulated receptor which is constitutively expressed in the activated state. For c-sis, the exact mechanism by which a normal growth factor-encoding gene has been converted into an oncogene remains unknown.

Further evidence implicates the c-myc gene in the control of cell proliferation; it is activated rapidly after stimulation of haematopoietic cells and fibroblasts by mitogens, PDGF^{4,5} and regenerating liver tissue⁶. Moreover, enhanced expression of c-myc can eliminate partly the PDGF requirement for cell growth⁷. Nuclear proteins such as myc, which are activated by growth factor stimulation, may function as transducing signals to effect the cellular response to growth factors. Here we report that quiescent murine fibroblasts respond to stimulation with serum or individual growth factors by rapidly synthesizing c-fos mRNA and protein; this is followed by the appearance of c-myc

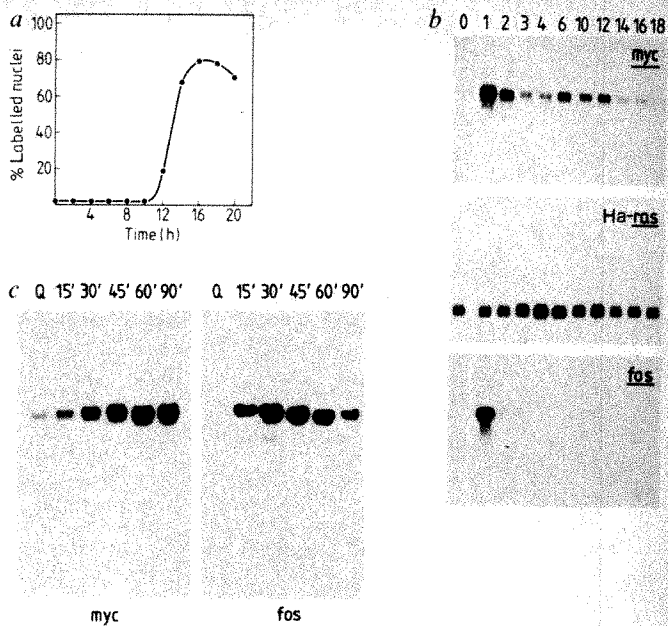
mRNA and the subsequent rapid degradation of almost all c-fos mRNA.

Induction of c-onc genes

To investigate the involvement of c-onc genes in the control of cellular proliferation, we analysed their expression following serum stimulation of quiescent NIH 3T3 cells (clone 7, from D. Lowy) stimulated after 3 days of serum deprivation with 20% fetal calf serum (FCS). This led to synchronous cell cycle initiation (Fig. 1a); the levels of c-Ha-ras mRNA (Fig. 1b) and c-Ki-ras mRNA (data not shown) increased no more than threefold in serum-stimulated cells at any of the time points analysed. Expression of c-myc, however, was induced more than 20-fold 1 h after stimulation with serum (Fig. 1b) followed by a slow decrease until reaching the basal level of quiescent cells after ~18 h (Fig. 1b). The change in c-fos expression was even more dramatic, increasing by approximately two orders of magnitude 1 h after serum stimulation and decreasing to basal levels 1 h later (Fig. 1b). Subsequent analyses showed that c-myc transcripts appeared at significantly elevated levels after 30 min of stimulation and reached a maximum after 60 min. Induction of c-fos clearly preceded the activation of c-myc mRNA expression (Fig. 1c) and was detectable as early as 5 min after stimulation (data not shown). It should be noted, however, that the type of RNA analysis used here measures steady-state levels,

Fig. 1 Time course of *c-myc* mRNA expression in quiescent NIH 3T3 cells stimulated with FCS. *a*, Thymidine labelling index following FCS stimulation. The number of labelled nuclei is plotted against time after addition of medium supplemented with 10% FCS. *b*, Time course of *c-myc*, *c-Ha-ras* and *c-fos* expression following FCS stimulation. Sizes of mRNAs are 2.3 kilobases (kb) (*c-myc*), 1.4 kb (*Ha-ras*) and 2.2 kb (*c-fos*) in this and all subsequent figures; 4 μ g poly(A⁺) RNA were analysed per lane. *c*, Time course of *c-myc* and *c-fos* expression following FCS stimulation; 15 μ g total RNA were applied to each lane.

Methods: *a*, NIH 3T3 cells were plated in 35-mm dishes containing 3 ml of medium supplemented with 10% FCS. When the cells were ~70% confluent, the medium was replaced with Dulbecco's modified Eagle's medium (DMEM) containing 0.5% FCS. After 2-3 days, cells were stimulated by adding medium supplemented with 20% FCS. Cells were labelled at 2-h intervals for 2 h with 1 μ Ci ³H-thymidine ml⁻¹. About 500 nuclei were counted in duplicate in each case. *b*, RNA was isolated from cells grown in 600-cm² dishes, selected for poly(A⁺) RNA, separated on formaldehyde-agarose gels and analysed by Northern blotting as described elsewhere^{15,21}. The oncogene-specific probes used were *v-fos* (1.1-kb *Pst*I-*Bgl*II fragment)²², *v-Ha-ras* (BS9, obtained from R. Ellis)²³ and mouse *c-myc* (provided by M. Cole)²⁴.



so we cannot distinguish whether the dramatic increase in the level of *c-fos* mRNA and its rapid disappearance are the result only of a modulated rate of transcription or whether it reflects also modulation of *c-fos* mRNA stability. Transcripts from four other proto-oncogenes tested were either not detectable (*c-sis* and *c-fms*) or did not show changes in the level of expression (*c-abl* and *c-raf*) (data not shown).

Induction by growth factors

Serum contains many growth factors, hormones and other growth-supporting components. We therefore analysed the induction of *c-fos* and *c-myc* expression by the purified growth factors EGF, fibroblast growth factor (FGF) and PDGF, each of which can induce a new round of DNA synthesis when added to quiescent NIH 3T3 cells in serum-free medium. Both FGF and PDGF are efficient in 80-90% of the cells, whereas EGF stimulation leads to cell-cycle initiation in only 50% of the cells (Fig. 2a).

The strong initial induction of *c-myc* expression by FCS was reproduced using either EGF, FGF or PDGF alone (Fig. 2b). Expression of *c-myc*, however, was considerably more stable in FGF- or FCS-treated cells than in EGF- or PDGF-stimulated cells, where the concentration of *c-myc* mRNA decreased to

relatively low levels 2 h after stimulation. The expression of *c-fos* dramatically increased 1 h after stimulation of quiescent NIH 3T3 cells with FGF or PDGF and was then abolished rapidly (similar to FCS-stimulated cells) (Fig. 2b). In contrast, EGF-treated cells showed a considerably lower induction of *c-fos* mRNA expression (Fig. 2b), possibly reflecting the weaker mitogenic effect of EGF on NIH 3T3 cells compared with FGF or PDGF (Fig. 2a). In contrast to *c-myc* and *c-fos*, no changes in *c-Ha-ras* expression were observed after stimulation with EGF, FGF or PDGF (Fig. 2b). These results clearly show that the expression of *c-myc* and *c-fos* in NIH 3T3 cells is rapidly and specifically inducible by purified growth factors. However, a crude preparation of FGF was used in this study, and thus may contain impurities that could enhance its effect on NIH 3T3 cells. Induction of a *c-fos*-related gene (*r-fos*) by PDGF has been observed also (C. Stiles, personal communication).

Superinduction by cycloheximide

Cultivation of quiescent NIH 3T3 cells (in 0.5% FCS) for 4 h with the protein synthesis inhibitor cycloheximide resulted in a slightly increased expression of both *c-myc* and *c-fos* mRNA (Fig. 3). Moreover, stimulation of quiescent NIH 3T3 cells for 4 h with 20% FCS and cycloheximide led to the accumulation

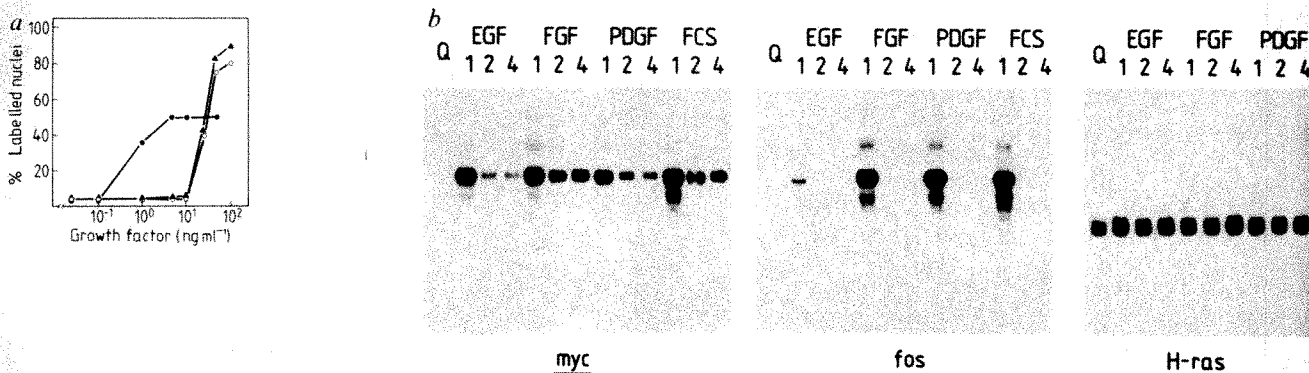


Fig. 2 Induction of *c-myc* and *c-fos* mRNA expression by growth factors. *a*, Thymidine labelling index of NIH 3T3 cells stimulated for 24 h by growth factors. *b*, Northern blot analysis of *c-myc*, *c-fos* and *c-Ha-ras* expression in NIH 3T3 cells in a quiescent growth state (Q), and 1, 2 and 4 h after growth-factor stimulation; 15 μ g total RNZ applied to each lane.

Methods: *a*, Quiescent cells (see Fig. 1 legend) were stimulated with DMEM containing the indicated doses of either EGF (●), FGF (○) or PDGF (△) and labelled for 24 h with 1 μ Ci ml⁻¹ of ³H-thymidine before being processed for autoradiography. *b*, Quiescent cells stimulated with DMEM containing either EGF (10 ng ml⁻¹; BRL), FGF (100 ng ml⁻¹; BRL) or PDGF (100 ng ml⁻¹; BRL) (similar results were obtained with highly purified PDGF), for different periods as indicated.

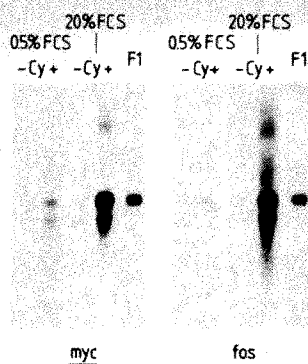


Fig. 3 Superinduction of *c-myc* and *c-fos* mRNA expression. Quiescent NIH 3T3 cells (see Fig. 1 legend) were cultured for 4 h in either 0.5% or 20% FCS with (+ Cy) or without (- Cy) cycloheximide ($5 \mu\text{g ml}^{-1}$). F1, NIH 3T3 cells 1 h after stimulation with serum; 15 μg total RNA were analysed as described in Fig. 1 legend. Left-hand lanes (0.5% FCS) were exposed for 15 h, right-hand lanes (20% FCS) for 2 h.

of *c-myc* and *c-fos* RNA to concentrations several fold higher than the maximum observed in the absence of cycloheximide (Fig. 3, lane F1). Hence, *c-myc* and *c-fos* genes may be modulated (repressed) by an unstable protein or by autoregulation and *c-fos* mRNA seems to be considerably more stable in the presence of cycloheximide, presumably because of the absence of a protein (or proteins) that would normally cause rapid degradation following the peak level of expression 1 h after stimulation. Superinduction of *c-myc* expression by cycloheximide has been observed previously in other cells⁴ and in regenerating liver tissue⁶.

Increased expression in proliferating cells

The rapid induction of *c-fos* and *c-myc* expression after growth-factor stimulation suggests that these proto-oncogenes have a primary role in the control of cell proliferation. We therefore examined their expression in rapidly proliferating and quiescent cells. We found increased expression of both *c-myc* and *c-fos* mRNA in growing relative to quiescent cells; the highest concentration of expression was detected in transformed cells (Fig. 4). Thus, there is a correlation between the expression of *c-fos* and *c-myc* proto-oncogenes and cellular proliferative activity. Although expression of *c-myc* in transformed cells approached that in serum-stimulated (1 h) NIH 3T3 cells, the level of *c-fos* mRNA was considerably lower in the transformed cells than in stimulated normal cells (data not shown).

Synthesis of *c-fos* protein

As growth-factor stimulation of NIH 3T3 cells leads to a dramatic induction of *c-fos* mRNA, we investigated *c-fos* protein synthesis in these conditions. Synthesis of the *fos* gene product was measured by immunoprecipitation in both normal mouse fibroblast (NIH 3T3) and normal rat fibroblast (208F)⁸ cell lines. Almost undetectable levels of *c-fos* protein occurred in serum-deprived cells, but 1 h after stimulation we found a dramatic induction of a highly modified relative molecular mass (M_r) 62,000 form of the *c-fos* protein. At 2 h post-stimulation, *fos* protein synthesis was already reduced ~fivefold, the time course of *fos* protein synthesis (Fig. 5a) reflecting the previously observed pattern of mRNA induction (Fig. 1b). In addition to the *fos* protein, p39, the cellular protein complexed to *fos*^{9,10}, was also detected.

In agreement with our RNA data, *c-fos* protein induction by FGF and PDGF was equivalent, whereas induction by EGF

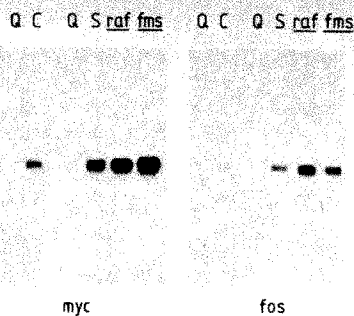


Fig. 4 Increased levels of *c-myc* and *c-fos* mRNA in growing versus quiescent cells. Quiescent NIH 3T3 cells (Q) were compared with normally proliferating non-synchronized NIH 3T3 cells growing in 10% FCS (C); 15 μg total RNA were analysed in this experiment. Expression of *c-myc* and *c-fos* was also analysed in quiescent NIH 3T3 cells (Q) relative to spontaneously transformed NIH 3T3 cells (S) or NIH 3T3 cells transformed by either long terminal repeat-activated *c-raf* gene (*raf*)²⁵ or the McDonough strain of feline sarcoma virus (*fms*)²⁶; 4 μg poly(A⁺) RNA were applied to each lane. Equal amounts of RNA were loaded as determined by ethidium bromide staining of the gel. Northern blot analyses were carried out as described in Fig. 1 legend.

was marginal. However, 10% FCs or the combination of EGF, PDGF and FGF gave stronger stimulation than any of the growth factors alone (Fig. 5b). Using antibodies directed against *myc*-specific synthetic peptides (from Dr Richard Chizzonite), we observed induction also of the *c-myc* protein in NIH 3T3 cells 1 h after stimulation by 10% FCS, FGF, PDGF but not by EGF (data not shown). However, the degree of induction and the amount of *c-myc* protein expressed at this time point was less than that observed with *c-fos*.

Modification of *c-fos* protein

In cells transformed by a *c-fos*-expressing plasmid, the *c-fos* product appears primarily as a M_r 55,000 protein on SDS-polyacrylamide gels during a 20 min pulse-labelling period. This protein is converted to short-lived high M_r forms of 57,000, 60,000 and 62,000 over 2 h (ref. 11). In normal mouse amniotic cells, which express constitutively a high level of *c-fos*, these highly-modified forms appear to be more stable¹¹. In serum-stimulated normal fibroblasts, the majority of the *c-fos* protein was in a highly modified form even in a 20 min pulse-labelling period (Fig. 5a, b). Pulse-chase analyses showed that the small amounts of M_r 55,000 and 57,000 forms detectable after a 15 min labelling period were converted rapidly to the larger forms in 15–30 min of subsequent chase. The profile of *c-fos* protein expression in NIH 3T3 cells and 208F cells was identical. During the 2-day serum starvation period, NIH 3T3 cells become quiescent whereas 208F cells still show a thymidine-labelling index of 10% (data not shown). Thus, *c-fos* induction is not dependent on cells being in the quiescent state. Modification of the *c-fos* protein continued during the chase periods and a stable mature form of M_r 62,000 was generated (p62^{c-fos}), in marked contrast to the pattern of *c-fos* protein modification detected in transformed cells. In these cells the M_r 62,000 form is detected only after 2 h of chase and is very short-lived¹¹.

Immunofluorescence studies

The relatively stable nature of the induced *c-fos* protein indicates that it should be detectable by immunofluorescence staining, which allows a more accurate examination of the time course of appearance of *c-fos* protein as cells are rapidly fixed at each time point. One hour after serum stimulation, high concentrations of *c-fos* protein appeared in the nucleus of NIH 3T3 cells (Fig. 6a). A granular staining pattern occurred throughout the

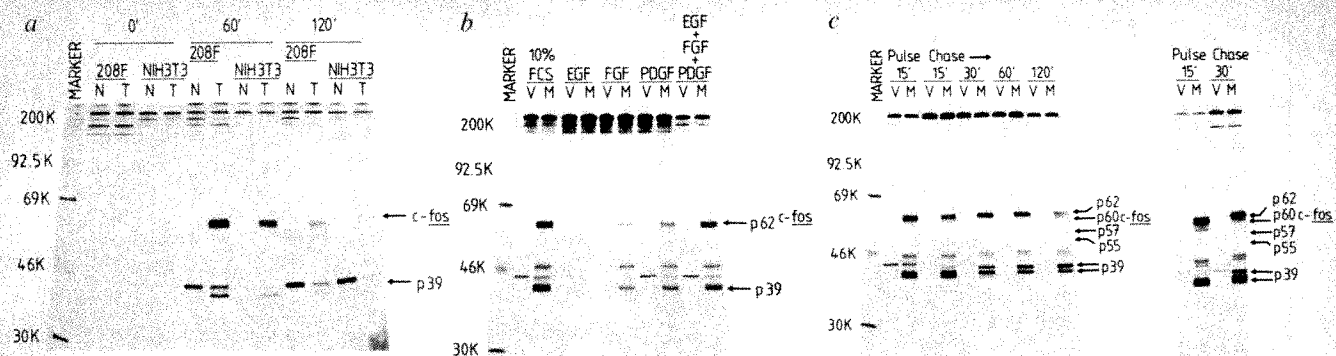


Fig. 5 Induction of *c-fos* protein synthesis by serum and growth factors. **a**, Normal 208F rat fibroblast cells and NIH 3T3 mouse fibroblast cells were stimulated for 0, 1 and 2 h with 10% FCS. Cultures were labelled with ^{35}S -methionine for 20 min at each time point. Cell extracts were prepared and immunoprecipitated with tumour-bearing rat serum (T) and with normal rat serum as control (N). Samples were analysed by electrophoresis on SDS-polyacrylamide gels. The numbers on the left indicate the molecular weights of the ^{14}C -methylated marker proteins (Amersham). For 208F cells each lane represents the immunoprecipitate from 1×10^7 c.p.m. of trichloroacetic acid (TCA)-insoluble protein and for NIH 3T3 cells, the immunoprecipitate from 5×10^6 c.p.m. of TCA-insoluble protein. **b**, NIH 3T3 cells stimulated for 1 h with 10% FCS, 50 ng ml $^{-1}$ EGF, 50 ng ml $^{-1}$ FGF and 50 ng ml $^{-1}$ PDGF were labelled for 15 min with ^{35}S -methionine. Extracts were prepared and immunoprecipitated with V (*v-fos* protein-specific) and M (*fos* protein-specific) peptide antisera. Each lane represents the immunoprecipitate from 10^6 c.p.m. of TCA-insoluble protein. **c**, Pulse-chase analysis of *fos* protein synthesis in NIH 3T3 and 208F cells. Cultures were stimulated for 1 h with 10% FCS, then labelled for 15 min with ^{35}S -methionine. Following the labelling period, medium was removed and replaced with DMEM supplemented with 10% FCS, and cultures were incubated further. NIH 3T3 cell extracts were prepared after the 15-min labelling period and at 15, 30, 60 and 120 min of chase. 208F cell extracts were prepared after the 15-min labelling period and after 30 min of chase. Extracts were immunoprecipitated with affinity-purified *fos*-specific peptide antiserum (M), and as control an affinity-purified peptide antiserum specific for the *v-fos* protein which does not recognize the *c-fos* gene product (V).

Methods: Cell culture was done as described in Fig. 1 legend. For ^{35}S -methionine incorporation, cells were preincubated in 1 ml of methionine-free medium for 20 min before the labelling period. For serum stimulation, this medium was supplemented with 10% dialysed calf serum (Gibco); for growth-factor stimulation the medium was supplemented with the appropriate amount of the relevant growth factor. ^{35}S -methionine labelling was performed by adding 250 μCi ^{35}S -methionine ($\sim 800 \mu\text{Ci mmol}^{-1}$; Amersham). Preparation of cell extracts, immunoprecipitation and polyacrylamide gel electrophoresis were as described previously¹¹. Characterization of tumour-bearing rat sera as well as the V and M *fos* peptide antisera have been described elsewhere^{10,27}.

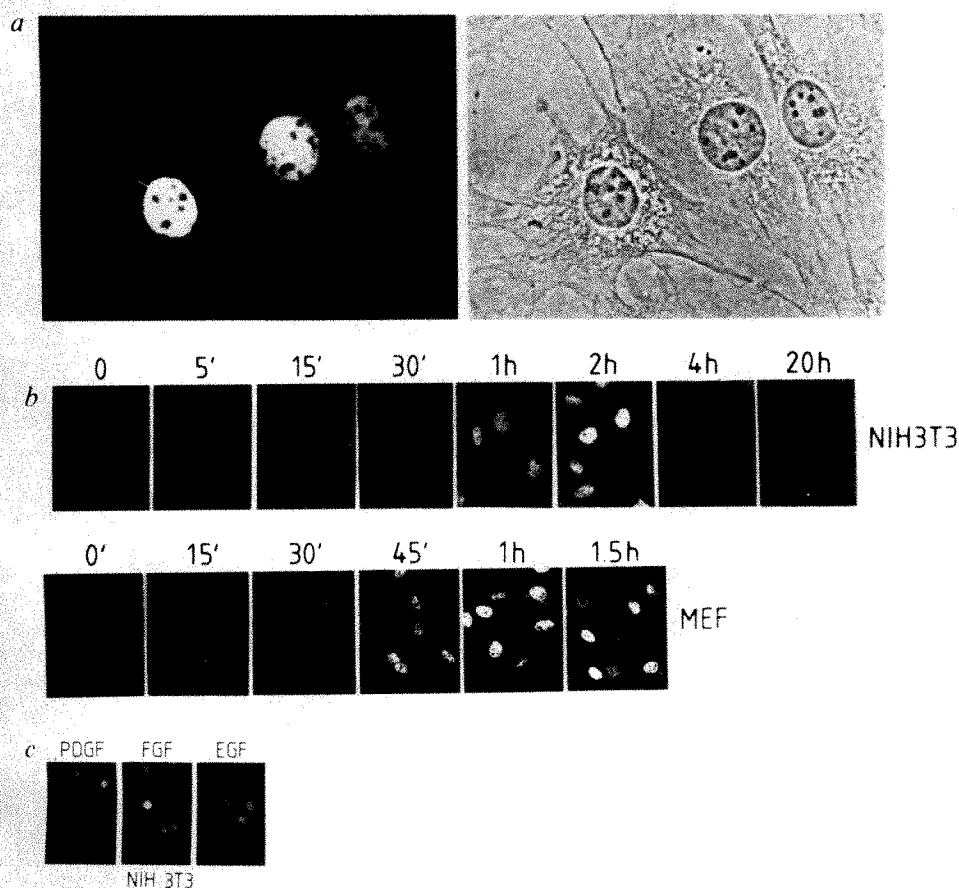


Fig. 6 Detection of *fos* protein induction by immunofluorescence staining. **a**, NIH 3T3 cells were stimulated for 2 h with 10% FCS (see Fig. 1 legend). Cells were prepared for immunofluorescence as described previously¹¹ and stained with TBRS followed by rhodamine-conjugated rabbit anti-rat IgG as the second antibody. Cells were photographed through a $\times 63$ lens (Leitz). Left panel, fluorescence; right panel, phase-contrast. **b**, Time course of *fos* protein induction analysed by indirect immunofluorescence. NIH 3T3 cells were induced by 10% FCS and cultures prepared for immunofluorescence at 0, 5, 15 and 30 min and 1, 2, 4 and 20 h post-stimulation. Tertiary MEFs were also stimulated with serum and prepared for immunofluorescence at 0, 15, 30, 45, 60 and 90 min; antisera were the same as in a. c, NIH 3T3 cells induced for 1 h with 50 ng ml $^{-1}$ each of PDGF, FGF and EGF were stained for *fos* expression as described for a. Cell culture was as described in Fig. 1 legend. Cells were fixed with 3% paraformaldehyde and permeabilized with 1% Triton X-100 as described previously¹¹. TBRS and rhodamine-conjugated rabbit anti-rat IgG (Cappel) were used at 1:100 dilutions as described elsewhere¹¹.

nucleus, except the nucleoli. A similar time course of *c-fos* protein induction was observed with NIH 3T3 cells, 208F cells and tertiary mouse embryo fibroblasts (MEFs) (Fig. 6b; data on 208F cells not shown). A small amount of *c-fos* protein was detected in cell nuclei in 5 min (Fig. 6b). Maximal fluorescence was observed at 1–2 h post-induction; it was drastically reduced in ~4 h and undetectable after 20 h. The demonstration that MEF cells also activate *c-fos* expression following stimulation shows that induction is not an artefact of established cell lines.

Immunofluorescence staining of NIH 3T3 cells following induction by EGF, PDGF and FGF (Fig. 6c) confirmed the conclusions from the RNA analyses (Fig. 2b) and immunoprecipitation experiment (Fig. 5b) that PDGF and FGF were more efficient than EGF at stimulating *c-fos* expression (Fig. 5c). Similar observations were made when growth factor-stimulated MEFs were analysed by immunofluorescence (data not shown). In general, stimulated cells gave the same overall appearance of fairly uniform staining independent of the agent used.

Cell proliferation and *c-onc* expression

The very rapid appearance of *c-fos* mRNA and protein after growth factor stimulation argues strongly that *c-fos* activation affects gene expression by primary growth factor, whereas activation of *c-myc* may be a later event. The sudden disappearance of *c-fos* mRNA also contrasts strikingly with the gradual decline of *c-myc* mRNA. The superinduction of both mRNAs by cycloheximide suggests that the levels of *c-fos* and *c-myc* mRNA are in part regulated by the action of a newly-synthesized gene product. Previously, it has been observed that the 3'-untranslated region of the *c-fos* gene is involved in inhibition or expression of the regulation of *c-fos* mRNA stability¹². It is possible that these sequences of the *c-fos* gene or its mRNA are recognized by the regulatory proteins.

Serum induction of fibroblasts results in the presence of high levels of *c-fos* protein for at least 2 h (Fig. 6b). If such *c-fos* stimulation is necessary for the normal cell cycle, a small population of cells which express a high concentration of the *c-fos* protein would be expected, but this is not the case (T.C., unpublished data). Such high expression does not seem to be a normal condition of proliferating fibroblasts in cell culture, but rather is a direct consequence of the growth factor-receptor interaction. Experiments are in progress to determine whether *c-fos* expression is involved in the transition from the G₀ to G₁ phase of the cell cycle rather than in the induction of DNA synthesis.

Implications for transformation

Induction of *c-fos* results in high concentrations of *c-fos* protein expression equivalent to that observed in *c-fos*- or *v-fos*-transformed cells for a period of 2–4 h, but the induced cells maintain a normal morphological phenotype. Thus, *c-fos* protein must be expressed for an extended period of time or even constitutively (such as in virus-infected cells) to induce transformation. Alternatively, as the induced *fes* protein is immediately exten-

sively modified, modified *c-fos* protein may not be capable of inducing transformation. The deletion in the *v-fos* gene seems to prevent modification¹¹, the p75^{gag-fos-fox} (ref. 13) transforming protein of FBR-MSV is not modified, and modification of the *c-fos* protein in cells transformed by the MMV plasmid is not efficient¹¹. It is possible, therefore, that the extensive post-translational modification of the *c-fos* product is a regulatory mechanism which affects its ability to induce cellular transformation.

Role of *c-fos*

Previous analyses of tissues and non-synchronized primary cell cultures have shown that *c-fos* is expressed at high concentrations specifically in cells constituting the fetal membranes and placenta¹⁴, bone marrow¹⁵, early fetal liver^{15,16} and differentiated macrophages^{15,16}. The observed stage-specific expression¹⁴ as well as studies using *in vitro* differentiation systems^{15,16} suggest a correlation between *c-fos* expression and differentiation. The expression of exogenous *c-fos* genes can trigger the differentiation of F9 teratocarcinoma stem cells¹⁷. Together, these findings suggest strongly that *c-fos* is involved in the induction of differentiation in particular cell types. In such cells, *c-fos* expression is indeed subject to a different mode of regulation compared with growth factor-stimulated fibroblasts. Thus, non-synchronized primary cultures of amniotic cells show a high concentration of expression in all the cells¹⁵. In addition, expression of *c-fos* in CSF-1 (colony-stimulating factor-1) stimulated macrophages shows a different pattern of cell cycle-related regulation and much less pronounced variations in the level of expression compared with fibroblasts (R.M. and L. Guilbert, unpublished results). This suggests that *c-fos*-expressing cells can be divided into at least two categories: (1) cells (for example, fibroblasts) which show a stringent control of *c-fos* expression and in which *c-fos* may have a key role in the control of cell proliferation and (2) cells (for example, amnion cells, macrophages) which express *c-fos* more or less constitutively and in which *c-fos* has a function in differentiation. The function of the *c-fos* gene product may be associated with the transduction of nuclear signals which, depending on the differentiated state of the cell, may affect regulatory pathways either involved in the control of proliferation or in the induction (or progression) of differentiation. In this context it is interesting to note that EGF has previously been reported to induce the expression of differentiation markers in certain cell types^{18,19}. These observations raise the intriguing question as to how the molecular mechanisms governing cellular proliferation and differentiation may be related to each other.

After submission of this manuscript, another study²⁰ was published reporting rapid induction of the *c-fos* gene following growth-factor stimulation of BALB/c/3T3 cells.

We thank E. F. Wagner for reading the manuscript, R. S. Lerman for PDGF, M. Cole and R. Ellis for mouse *c-myc* and *v-Ha-ras* plasmids, and B. Blanasch for help in preparation of the manuscript.

Received 19 September; accepted 7 November 1984.

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Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity

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A chemically-synthesized gene and natural complementary DNA coding for human lymphotoxin were isolated and engineered for expression in Escherichia coli. Purified recombinant lymphotoxin shows cytotoxic activity on murine and human tumour cell lines in vitro and causes necrosis of certain murine sarcomas in vivo.

LYMPHOTOXIN was first characterized as a biological factor in mitogen-stimulated lymphocytes having anticellular activity on neoplastic cell lines¹⁻³. It has a spectrum of associated cytotoxic activities, including cytostasis of certain tumour cell lines and marked cytolysis of other transformed cells⁴⁻⁸. On primary cell cultures and normal cell lines, lymphotoxin shows little or no anticellular activity. This discrimination led to *in vivo* studies⁹⁻¹¹ suggesting that lymphotoxin has a potent antitumour activity.

Purification and subsequent characterization of lymphotoxin has proved difficult because of the small amount of active protein secreted by primary lymphocyte cultures. We reported recently the isolation and characteristics of lymphotoxin produced by the human lymphoblastoid cell line RPMI-1788 (ref. 12). Antibodies prepared against RPMI-1788-derived lymphotoxin neutralize all of the cytolytic activity produced by non-adherent lymphocytes¹³, suggesting that lymphotoxin from both sources is identical. Lymphotoxin is a glycoprotein with a relative molecular mass (M_r) 60,000–70,000 measured by molecular sieve chromatography^{12,13}, whereas monomeric lymphotoxin has a M_r of 25,000 (ref. 14); previous values of 20,000 (ref. 12) probably represent a degradation product. We report here the construction and expression in *Escherichia coli* of a DNA encoding human lymphotoxin and some of the *in vitro* and *in vivo* biological activities of this anticellular lymphokine.

Lymphotoxin gene design

Cultures of RPMI-1788 cells were induced with the tumour-promoting agent 4 β -phorbol-12 β -myristate 13 α -acetate (PMA) and produced 500–1,000 lymphotoxin units per ml, as determined in the murine fibroblast L-929 assay^{13,15,16}. Lymphotoxin was purified as described previously¹² and migrated on SDS-polyacrylamide gels as two bands of M_r 25,000 (95% of material) and 20,000 (5% of material). Microsequencing^{17,18} by the Edman degradation technique yielded sequence information on the intact molecule and on fragments produced by various enzymatic and chemical cleavages. A contiguous sequence of 155 residues was determined, but the carboxy-terminal region was difficult to determine because of the limited availability of material and hydrophobic nature of this region¹⁴. Three carboxy-terminal residues were determined by carboxypeptidase digestion (-Phe-Ala-Leu-COOH), but several residues in this region remained unknown¹⁴.

We constructed a synthetic gene containing an ATG translational initiation codon followed by codons for the 155 residues determined by microsequencing. This design assumed that the unknown carboxy-terminal residues would not be necessary for lymphotoxin activity. The gene design incorporated *E. coli* codon bias^{19,20} and substituted preferred human codons²¹ where no *E. coli* bias was apparent. The entire gene was assembled in three segments from 58 individual oligodeoxynucleotides

synthesized by the solid phase phosphite method^{22,23}. Extracts prepared from *E. coli* cultures containing the resulting expression plasmid pLTXBtrp1 were not active in the L-929 assay, suggesting that the carboxy-terminal residues not identified by protein sequencing are necessary for lymphotoxin activity.

Lymphotoxin cDNA isolation

The synthetic lymphotoxin gene was used as a probe to isolate the natural lymphotoxin complementary DNA sequence. RNA was isolated from a culture of non-adherent human peripheral blood lymphocytes stimulated with PMA, staphylococcal enterotoxin B and thymosin α_1 (refs 13, 24). This RNA was used to prepare a cDNA library in vector λ gt10 (ref. 25), which was screened²⁶ in conditions of low stringency²⁷ with a probe prepared from the N-terminal coding region (116 base pairs, bp, in length) of the synthetic lymphotoxin gene.

The DNA sequence of the hybridizing phage containing the longest cDNA insert (λ LT11) was determined by the dideoxy-chain termination method (ref. 28; Fig. 1). There is an open reading frame of exact amino acid homology with the determined lymphotoxin protein sequence¹⁴. The lymphotoxin cDNA structure is similar to that of other cDNAs which have been characterized, containing 79 bp of 5' untranslated and 626 bp of 3' untranslated sequence and the consensus polyadenylation addition sequence AATAAA²⁹ just upstream of the poly(A) tail. λ LT11 cDNA encodes 34 residues before the sequenced amino terminus which have the characteristics of a signal sequence³⁰.

Table 1 Antigenic similarity of natural and recombinant lymphotoxin as assessed by neutralization of cytolytic activity

Lymphotoxin pretreatment	% Cytolysis	
	Natural lymphotoxin	Recombinant lymphotoxin
Preimmune serum	100	100
Rabbit anti-lymphotoxin	0	0
Lymphotoxin monoclonal antibody lymphotoxin-B	0	0
55 °C, 1 h	100	100
80 °C, 1 h	0	0

Ten units of lymphotoxin were preincubated with 5 μ l (5 μ g) polyclonal or monoclonal antibody in a total volume of 0.1 ml for 1 h at 37 °C before assay on murine L-929 cells as described previously^{13,15}. The assay is sensitive to 0.4 U in 0.1 ml. Preimmune and hyperimmune rabbit antisera were obtained from a rabbit immunized with purified natural lymphotoxin derived from RPMI-1788 cells. Neutralizing monoclonal antibody was derived from a mouse immunized with natural lymphotoxin; 1 mg of lymphotoxin-B neutralizes ~4,000 U of natural or recombinant lymphotoxin.

Fig. 2 Characterization of the gene and RNA coding for human lymphotoxin. Lanes 1-4, Southern hybridization of human genomic DNA digested with restriction endonucleases. Human lymphocyte DNA (5 μ g) prepared by the method of Blin and Stafford⁵⁴ was digested to completion with *Eco*RI (lane 1), *Pvu*II (lane 2), *Hind*III (lane 3) or *Pst*I (lane 4). The digested DNAs were electrophoresed on a 1% agarose gel and blotted to a nitrocellulose membrane³¹. A probe prepared⁵² from the coding region of the lymphotoxin cDNA was hybridized (10^7 c.p.m.) with the filter for 16 h and then washed as described elsewhere^{24,26}. Lanes 5-7, the ethidium bromide staining pattern of RNAs electrophoresed through a denaturing agarose gel. The 10- μ g RNA samples were electrophoresed through the gel (1.75% agarose, 0.025 M sodium citrate (pH 3.8), 6 M urea) for 7 h at 25 mA and 4 $^{\circ}$ C^{55,56}. The gel was stained in 5 μ g ml⁻¹ ethidium bromide and photographed with an ultraviolet light source, then washed in 10 mM sodium phosphate (pH 7.0) to remove urea. RNA was transferred to nitrocellulose⁵⁷ and then hybridized and washed as above for the Southern blot (lanes 8-10). The samples are mRNA from RPMI-1788 cells (lanes 5, 8), induced adherent leukocyte (macrophage) cell RNA (lanes 6, 9) and induced non-adherent (lymphocyte) mRNA (lanes 7, 10). The mobility of the band observed in lanes 8 and 10 corresponds to an RNA 14S in size. 28S and 18S refer to ribosomal RNA size markers; numbers on the left indicate mobilities of *Hind*III λ DNA fragments in kb.

Methods: A hybrid synthetic gene/natural cDNA expression plasmid was constructed as follows. A 685-bp fragment containing a *trp* promoter and DNA coding for 125 residues of the synthetic gene were isolated by digesting plasmid pLTXBtrpI with *EcoRI* and *PstI*. A 301-bp fragment containing DNA coding for the C-terminal 51 amino acids of lymphotoxin was isolated by digesting the subcloned cDNA of λ LTI with *EcoRI* and *PstI* (these sites are shown above at nucleotide positions 554 and 855). These fragments were isolated by electrophoresis on 5% polyacrylamide gels and electroelution. The fragments were ligated into *EcoRI*-cleaved pBR322 treated with bacterial alkaline phosphatase⁵³ to reduce background transformants. The resulting expression plasmid, pLTtrpI, was characterized by restriction endonuclease digestion and DNA sequencing.

We used ^{32}P -labelled lymphotoxin cDNA to analyse the structure of the lymphotoxin gene and RNA. Southern blots³¹ hybridized with the lymphotoxin cDNA probe suggest that human lymphotoxin is encoded by a single gene (Fig. 2). Northern hybridizations of RNAs from induced lymphocytes or RPMI-1788 cells demonstrate that a transcript of ~14S is produced from the lymphotoxin gene (Fig. 2) which agrees well with the length of the cDNA (1,329 bp).

Polyclonal antibodies prepared in rabbits against natural lymphotoxin¹³ neutralized all of the cytolytic activity contained in the recombinant cultures (Table 1). A murine monoclonal antibody (LT-B) derived to natural lymphotoxin (C.V.B. and T.S.B., unpublished results) is also capable of neutralizing completely the L-929 cytolytic activity of lymphotoxin from natural and recombinant sources (Table 1). The recombinant lymphotoxin

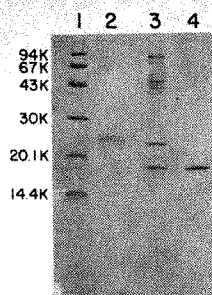


Fig. 3 Reducing (2-mercaptoethanol) 15% SDS-polyacrylamide gel of proteins stained with Coomassie blue. Lane 1, size markers (sizes ($M_r \times 10^3$) indicated to the left); lane 2, natural lymphotoxin purified as previously described¹²; lane 3, proteins from extracts of pLTp1-containing *E. coli* selectively precipitated with 40% saturated ammonium sulphate; lane 4, eluate from a Sepharose/monoclonal antibody lymphotoxin-B column. Conditions of immunoaffinity purification are briefly as follows: The monoclonal antibody purified from ascites fluid by ion exchange chromatography was coupled to cyanogen bromide-activated Sepharose at a concentration of 2 mg ml⁻¹ resin. A 20-ml column was equilibrated consecutively with TBS (0.05 M Tris-HCl, pH 7.0, 0.15 M sodium chloride, 2 mM EDTA); 0.1 M acetic acid, pH 4.5, 150 mM NaCl (elution buffer), then TBS. A 40% saturated ammonium sulphate precipitate of *E. coli* pLTp1 lysate was dissolved in 0.1 M Tris-HCl, pH 7.4 and 5 mM EDTA and loaded onto the column at a rate of one column volume per h. Following extensive washing with TBS containing 0.05% Tween-20, specifically-bound material was eluted and immediately adjusted to pH 7.8 with 0.1 volume 1 M Tris-HCl, pH 8.5 and stored at 4°C. The specific bioactivity of this purified lymphotoxin was 2.5×10^7 U mg⁻¹, where 1 U of lymphotoxin is defined as the amount required to achieve 50% lysis of 12,000 murine L-929 cells in a standard microtitre well.

activity also exhibited thermolability similar to natural lymphotoxin (ref. 16 and Table 1).

The recombinant lymphotoxin was purified on a monoclonal antibody (LT-B) column (Fig. 3). The mobility of the eluted lymphotoxin corresponds to M_r 18,000, consistent with the predicted value of 18,600 based on the deduced amino acid sequence. Recombinant lymphotoxin produced in *E. coli* clearly causes cytolysis of murine L-929 cells, similar to lymphotoxin derived from RPMI-1788 cells. Anticellular activity was measured *in vitro* by plating tumour cells at a low density and then monitoring their growth in the presence of lymphotoxin. We found that the recombinant and natural lymphotoxin had

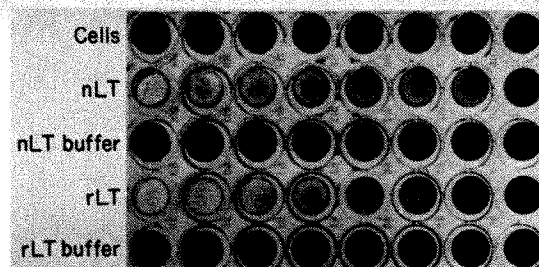


Fig. 4 Cytotoxic activity of recombinant and natural lymphotoxin on ME180 cells. Natural lymphotoxin (second row, nLT) and recombinant lymphotoxin (fourth row, rLT) were added to 96-well flat-bottom microtitre plates at 1×10^4 U per well and serially diluted twofold, across the plate from left to right. The final concentration in the far right well is 78 U. Buffer 1 and buffer 2 controls (as in Table 2) were added to the third and fifth rows, respectively. ME180 cells were subsequently added at a concentration of 5×10^4 per well and the plates incubated at 37°C. After 72 h the supernatants were removed and the cells stained with crystal violet. Results show significant cytotoxic activity of lymphotoxin on ME180 cells (second and fourth rows) when compared with buffer controls (third and fifth rows) and ME180 cells alone (top row).

a significant antiproliferative effect on the human tumour cell line ME-180, a human cervical carcinoma (Fig. 4). A normal lung fibroblast line, WI-38, was not sensitive to lymphotoxin (ref. 16).

Recombinant and natural lymphotoxin were tested in an *in vivo* tumour necrosis assay³² which involves growing methylcholanthrene-induced (MethA) sarcomas in susceptible mice and then injecting the tumours directly with lymphotoxin or control samples. After 20–24 h, the mice are killed and the tumours removed and histologically scored for the extent of necrosis³². Both recombinant and natural lymphotoxin caused significant necrosis of MethA sarcomas *in vivo* (Table 2).

Discussion

We report here the cloning and expression of DNA sequences encoding human lymphotoxin. The various M_r s of lymphotoxin described previously^{5,33,34} probably do not represent a family of closely-related proteins, because hybridization studies suggest that human lymphotoxin is encoded by a single gene (Fig. 2) and polyclonal or monoclonal antibodies neutralize all of the L-929 anticellular activity produced from RPMI-1788 cells and induced non-adherent lymphocyte cultures¹³. These various size species could result from differential processing of the lymphotoxin mRNA or protein, but a more probable explanation is that this heterogeneity results from aggregation and/or degradation phenomena. As determined by protein sequencing and cDNA cloning, mature lymphotoxin is 171 residues in length (M_r 18,660); N-linked glycosylation^{14,35} at the observed potential Asn-X-Ser site (residues 62–64 of Fig. 2a) probably accounts for the increased size (M_r 25,000) observed for natural lymphotoxin. The absence of carbohydrate on recombinant lymphotoxin does not seem to affect its cytotoxic activity, as the specific activity on L-929 cells of lymphotoxin produced by recombinant means (2.5 – 13×10^7 U mg⁻¹) is approximately that reported for natural lymphotoxin (4×10^7 U mg⁻¹)¹⁴. The 25,000 M_r form of lymphotoxin contains 23 extra N-terminal residues when compared with the 20,000 M_r lymphotoxin¹⁴. Production of lymphotoxin by recombinant methods should eliminate the heterogeneous nature of the lymphotoxin amino terminus and allow the isolation of lymphotoxin with a distinct amino terminal end.

The amino acid sequence of lymphotoxin was compared with the sequences of the lymphokines γ -interferon²⁴, interleukin-2 (ref. 36), and interleukin-3 (ref. 37). No significant homologies were observed even though all these lymphokines are secreted from induced lymphocytes and have similar sizes to lymphotoxin. There is at least one cytolytic factor of haematopoietic origin distinct from lymphotoxin, the cytokine tumour necrosis

Table 2 Necrosis of MethA sarcoma *in vivo* by recombinant and natural lymphotoxin

Treatment	Sarcoma necrosis score			
	+++	++	+	-
No. of mice				
Natural lymphotoxin, 25,000 U	4	-	-	-
Natural lymphotoxin, 10,000 U	4	-	-	-
Buffer 1 control	-	-	-	10
Recombinant lymphotoxin 200,000 U	14	2	2	-
Recombinant lymphotoxin, 25,000 U	3	-	-	1
Recombinant lymphotoxin, 10,000 U	3	-	1	-
Buffer 2 control	-	-	1	9

Tumour necrosis activity was assayed after intratumoral injection of lymphotoxin (or buffer control) into 7–10 day MethA transplants in CB6F1 mice. At 24 h after the injection, mice were killed and tumours scored histologically for haemorrhagic necrosis. Natural lymphotoxin was purified as previously described¹² and recombinant lymphotoxin was immunoaffinity purified (Fig. 3). Natural lymphotoxin was in buffer 1 (0.01 M Tris-HCl, 0.05 M (NH₄)₂ HCO₃, pH 8.0) and recombinant lymphotoxin was in buffer 2 (0.15 M NaCl, 0.1 M sodium acetate, 0.1 M Tris-HCl, pH 7.8). +++, Maximum response, 50–75% of the tumour mass markedly necrotic after 24 h; ++, moderate response, 25–50% haemorrhagic necrosis; +, minimal response of <25% haemorrhagic necrosis; -, no visible necrosis.

factor (TNF)^{32,38-41}. This factor causes necrosis of MethA(a) sarcoma *in vivo*; it is also active in causing lysis of murine L-929 cells *in vitro*^{32,39}. TNF activity on L-929 cells is not inhibited by neutralizing antibodies specific for lymphotoxin¹³, however, and appears to have distinct biochemical properties^{39,40}. Furthermore, the lymphotoxin cDNA probe failed to hybridize on Northern blots to mRNA from induced macrophages producing cytolytic activity (Fig. 2). The isolation of a TNF cDNA clone further demonstrates that lymphotoxin and TNF are distinct molecules (see accompanying article⁴²).

A cytolytic factor derived from B-cell lines which displays *in vitro* and *in vivo* anticellular activity has been described⁴³; this has been designated 'tumour necrosis factor' based on its activity in the MethA sarcoma assay. This activity probably results from lymphotoxin, however, because it has similar biological activities, biochemical properties and is made by the cell line (RPMI-1788) used in this study for the purification of lymphotoxin. Natural killer cells also can be induced to secrete an anticellular factor^{44,45}. The lymphotoxin gene probe and lymphotoxin-specific antibodies will be useful in determining the

relationship of this natural killer cell cytotoxic factor to lymphotoxin.

Lymphotoxin has been reported to act synergistically with α -interferon⁴⁶ and γ -interferon^{13,16,47} *in vitro* and *in vivo*. The potent antitumour activity of γ -interferon or lymphotoxin in natural preparations may be a result of the synergistic activity when both lymphokines are present¹³. The ready availability of lymphotoxin produced via recombinant methods will aid the biological characterization of this anticellular lymphokine. It will also help to define the antitumour mechanism of lymphotoxin, as well as its role *in vivo* in the regulation of the immune system and its interaction with other lymphokines.

We thank William Kohr and Rod Keck for protein sequencing studies; Dr Mark Matteucci for aid with the lymphotoxin synthetic gene design; the DNA Synthesis Group for preparation of oligomers; Dr Peter Seeburg for the λ gt10 vector; Dr David Goeddel for helpful suggestions, direction, and critical review of this manuscript; Irene Figari and Refaat Shalaby for tumour necrosis assays and the Bioassay Group for performing the murine L-929 assays.

Received 20 August; accepted 9 November 1984.

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Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin

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Human tumour necrosis factor has about 30% homology in its amino acid sequence with lymphotoxin, a lymphokine that has similar biological properties. Recombinant tumour necrosis factor can be obtained by expression of its complementary DNA in Escherichia coli and induces the haemorrhagic necrosis of transplanted methylcholanthrene-induced sarcomas in syngeneic mice.

TUMOUR necrosis factor (TNF) has been associated with *in vitro* and *in vivo* killing of tumour cells. This activity was discovered originally in the sera of mice and rabbits injected first with *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG) or other immunostimulatory agents, and subsequently

with endotoxin^{1,2}. Serum from such animals causes haemorrhagic necrosis and in some cases complete regression of certain transplanted tumours in mice^{1,2}. TNF-like activity has also been detected in the media of BCG/endotoxin-induced monocyte cultures (reviewed in ref. 2) and mitogen-stimulated peripheral

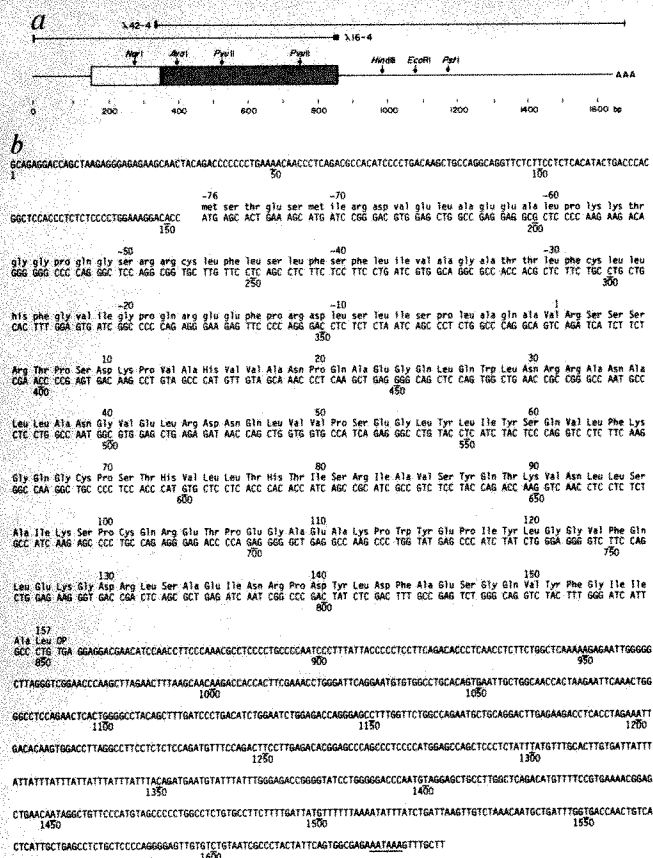


Fig. 1 TNF cDNA sequences and predicted amino acid sequence.

a, Schematic representation of human TNF cDNA clones. Overlapping clones λ 42-4 and λ 16-4 used in sequence determination and a schematic diagram of the complete cDNA structure are shown. Line, untranslated sequences; boxes, coding sequences; white portion, sequences encoding the signal peptide; shaded regions code for mature TNF. The black box on the 3' end of clone λ 16-4 indicates that this clone was obtained by specific priming. **b**, Nucleotide sequence and deduced amino acid sequence of human TNF cDNA. Numbers above each line refer to amino acid positions and numbers below each line refer to nucleotide positions. The amino acid labelled 1 represents the first amino acid of mature TNF¹⁶. The 76 amino acids preceding this position are indicated by lower case lettering. Sequence underlined indicates the polyadenylation recognition site²⁷.

Methods: **a**, Total RNA was extracted²⁰ from HL-60 cultures 4 h after PMA induction and poly(A)-containing RNA was purified on oligo(dT)-cellulose⁴³. Double-stranded cDNA was prepared by oligo(dT) priming¹⁹ using 7.5 μ g mRNA and fractionated on a 6% polyacrylamide gel. 700 ng cDNA >600 bp was recovered by electroelution. Synthetic EcoRI adaptors⁴⁴ were ligated to 20 ng cDNA before ligating into λ gt10 (ref. 26). 200,000 cDNA clones were obtained. The same conditions were used to prepare a specifically-primed cDNA library of 200,000 clones using as primer the hexadecanucleotide dTGGATGTTTCGCTCTCC (complementary to nucleotides 855–870). Plaque screening⁴⁵, ³²P-radiolabelling of synthetic 42-mer probe²⁵ and hybridizations²² were performed. **b**, DNA sequencing was performed by the dideoxynucleotide chain termination procedure^{46–48}. The cDNA insert of λ 42-4 consists of nucleotides 337–1643 and the cDNA insert of λ 16-4 consists of nucleotides 1–870.

blood leukocytes (PBLs)³.

TNF activity is cytolytic or cytostatic against many transformed cell lines *in vitro* without obvious species specificity, yet has no known effect on normal mouse embryo fibroblasts or non-transformed cell lines^{1,2,4–8}. Activated macrophages may constitute the major cellular origin of TNF^{1,5,9,10}, providing an important criterion for distinguishing this factor from the lymphoid cell-derived cytotoxin, lymphotoxin¹¹. The primary structure of lymphotoxin was determined recently by protein sequencing¹² and complementary DNA cloning (see accompanying article¹³).

Table 1 Human TNF production by various cell populations and cell lines

Cell source	Inducing agent(s)	Cytotoxic activity (U ml ⁻¹)	
		TNF	Lymphotoxin
Unfractionated PBLs	None	<8	<8
	LPS	20	<8
	BCG	82	<8
	BCG/LPS	86	<8
	BCG/LPS/PMA	140	<8
PBLs (adherent cells)	PMA	280	<8
	SEB/T α_1	100	10
	SEB/T α_1 /PMA	1,600	200
	None	<8	<8
	BCG/LPS	350	<8
PBLs (non-adherent cells)	SEB/T α_1 /PMA	590	<8
	None	<8	<8
	BCG/LPS	<8	<8
HL-60	SEB/T α_1 /PMA	<8	350
	None	<8	<8
U-937	PMA	380	<8
	None	<8	<8
	PMA	32	<8

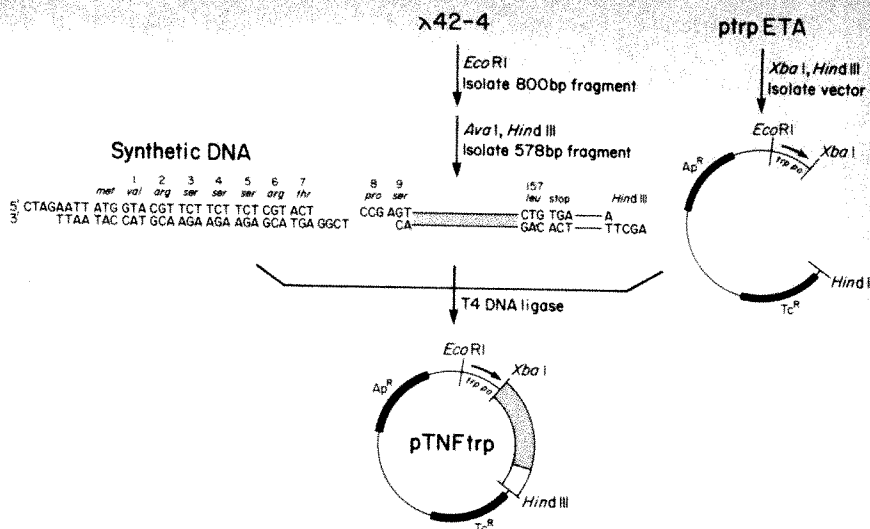
PBLs were obtained from plateletpheresis residues (Boston Red Cross) by Ficoll-Hypaque centrifugation³. Separation of PBLs into adherent and non-adherent populations was performed as described previously⁴¹. HL-60 (CCL 240) and U-937 cell lines (CRL 1593) were obtained from the American Type Culture Collection. Cells were suspended at 5×10^6 cells ml⁻¹ in RPMI 1640 media containing 10% fetal bovine serum. Cultures were induced with one or more of the following agents: 2×10^5 organisms per ml of BCG (Calbiochem-Behring), 20μ g ml⁻¹ *Salmonella typhimurium* lipopolysaccharide (LPS, Sigma), 1μ g ml⁻¹ staphylococcal enterotoxin B (SEB, Sigma), 1μ g ml⁻¹ thymosin α_1 (T α_1)⁴² and 10 ng ml^{-1} PMA (P-L Biochemicals). Cell-free supernatants were collected 24 h after induction except for the BCG/LPS and BCG/LPS/PMA treatments; for these two inductions a 24-h BCG stimulation was followed by an additional 24-h treatment with LPS and LPS/PMA, respectively. Samples were assayed for cytotoxic activity on mouse L-929 fibroblasts as described previously¹¹. The activities shown represent TNF-specific or lymphotoxin-specific units as determined after antibody neutralization at 4°C for 4 h before assay. The units indicated were obtained from one representative donor in the case of the PBLs and from a single experiment when cell lines were used. Rabbit anti-human TNF antiserum was prepared against partially purified TNF from PBLs (L. Svedersky and T. Bringman, unpublished results). Rabbit anti-human lymphotoxin antiserum was prepared against purified human lymphotoxin from RPMI 1788 lymphoblastoid cells¹¹.

Here we identify a cell line with monocyte-like characteristics providing a source for human TNF and its messenger RNA. cDNA clones were isolated that encode a polypeptide related structurally to lymphotoxin. This cDNA was engineered to direct the synthesis of a relative molecular mass (M_r) 17,000 protein in *E. coli* with the immunological characteristics as well as *in vitro* and *in vivo* biological properties of natural human TNF.

A human TNF-producing cell line

We isolated PBLs by Ficoll-Hypaque density centrifugation and fractionated them into adherent monocytic and non-adherent lymphocytic fractions. After stimulation with BCG and endotoxin (lipopolysaccharide, LPS), we detected an activity cytotoxic to murine L-929 cells in the culture media of unfractionated mononuclear cells and monocytes (Table 1). No cytotoxic activity was produced by the non-adherent cells following the same BCG/LPS induction procedure. The failure of rabbit anti-human lymphotoxin antibodies to neutralize the cytotoxic activity demonstrates its difference from lymphotoxin. Moreover, the results of previous *in vivo* studies using BCG/LPS induction procedures^{1,2} demonstrate that the activity can probably be attributed to TNF. Antiserum raised against partially purified PBL-produced TNF completely neutralized this activity (Table 1).

Fig. 2 Construction of a plasmid coding for the direct expression of mature human TNF in *E. coli*. The recombinant phage λ 42-4 (10 μ g) was digested with *Eco*RI and the 800-bp fragment containing the entire TNF coding region was isolated. TNF digestion with *Ava*I and *Hind*III gave a 578-bp fragment coding for amino acids 8–157. Two synthetic complementary deoxyoligonucleotides¹⁷, 5'-dCTAGAAT-TATGGTACGTTCTTCTCTCGTACT and 5'-dTCGGAGTACGAGAAGAAGAACGTA-CCATAAT, were designed to code for amino acids 1–7 of TNF, preceded by an ATG translational initiation codon, and to contain an *Xba*I cohesive terminus. The choice of codons for the first six amino acids of TNF was based on *E. coli* codon usage preferences⁴⁹. An AATT sequence was incorporated upstream of the ATG to maximize expression by giving optimal spacing between the initiation codon and the *trp* leader Shine–Dalgarno sequence⁵⁰. The pBR322-derived plasmid ptrpETA⁵¹ was cleaved with *Hind*III and *Xba*I and the large fragment recovered by electroelution. The *Ava*I–*Hind*III fragment and the two synthetic deoxyoligonucleotides were inserted into the plasmid pTNFtrp expression vector to give the plasmid pTNFtrp. The methods used to assemble the fragments and verify the construction of pTNFtrp have been described previously^{19,20}. *E. coli* W3110/pTNFtrp was grown in M9 medium containing 5 μ g ml⁻¹ tetracycline to 0.2 A_{550} units. Indole acrylic acid was added to a concentration of 20 μ g ml⁻¹. The cells were collected at A_{550} = 1.0 and washed with cold PBS. The final cell pellet was resuspended in 1 ml PBS, sonicated on ice for 30 s and the resulting extract diluted in PBS for assay on L-929 cells¹¹.



Yields of adherent cells from peripheral blood were low and the levels of TNF produced were variable and donor-dependent; we therefore tested alternative induction schemes for the production of TNF from total PBLs (Table 1). An increase in cytotoxic activity was observed when the PBLs were co-stimulated with *Staphylococcus* enterotoxin B, desacetyl-thymosin- α_1 and the tumour-promoting agent 4 β -phorbol 12 β -myristate 13 α -acetate (PMA). However, antibody neutralization experiments demonstrated that a significant portion of measured activity was lymphotoxin. Therefore, we screened a number of transformed cell lines of haematopoietic origin for their ability to synthesize TNF. Activity which could be neutralized by anti-TNF antiserum was detected following PMA treatment in two monocytic-like cell lines, HL-60, derived from a promyelocytic leukaemia¹⁴, and U-937, derived from a histiocytic lymphoma¹⁵ (Table 1). The HL-60 cell line consistently produced higher TNF titres (300–400 U ml⁻¹ 24 h after induction) than the U-937 cell line (<100 U ml⁻¹). A time course of TNF synthesis by HL-60 cultures indicated that measurable activity was detected 2 h after PMA treatment (data not shown). Therefore the HL-60 cell line was selected for future experiments; supernatants were collected 16–24 h after induction for protein purification and 4-h inductions were used when cells were collected for RNA isolation.

TNF cDNA clone identification

Human TNF was purified to homogeneity from filtrates of PMA-stimulated HL-60 cell cultures (see ref. 16). A single component of M_r 17,000 was observed when the purified TNF was analysed by SDS-gel electrophoresis in reducing conditions. To obtain amino acid sequence information, tryptic peptides of TNF were prepared and separated by reverse-phase HPLC.

The preliminary sequence Glu-Thr-Pro-Glu-Gly-Ala-Glu-Ala-Lys-Pro-Trp-Tyr-Glu-Lys was determined for the first tryptic fragment (TD-6) analysed. A single synthetic 42-base long deoxyoligonucleotide (42-mer) which could code for this amino acid sequence was chemically synthesized¹⁷ for use as a hybridization probe. The design of the probe sequence (dGAAACCCCT-GAAGGGGCTGAAGCCAAGCCCTGGTATGAAAAG) was based on published human codon usage frequencies¹⁸ and the codon bias of human γ -interferon¹⁹, tissue-type plasminogen activator²⁰ and lymphotoxin¹³. The general usefulness of this 'long probe' approach has been demonstrated recently by the identification of several cloned genomic DNA sequences^{21–23} and cDNAs^{24,25}.

An oligo(dT)-primed HL-60 cDNA library of ~200,000 clones prepared in λ gt10 (ref. 26) was screened with the ³²P-labelled 42-mer. The nine distinct phage which gave positive signals with this probe were hybridized with 'induced' and 'non-induced' ³²P-labelled cDNA probes¹⁹ prepared using poly(A) mRNA obtained from 4-h PMA-treated and untreated HL-60 cultures, respectively. Seven of these recombinant phage DNAs hybridized weakly to the induced probe but did not hybridize to the uninduced probe, as expected for authentic TNF cDNAs. Restriction endonuclease mapping indicated that these seven cDNA clones were related to each other and that the phage λ 42-4 contained the longest cDNA insert.

TNF cDNA sequence

We determined the sequence of the 1,300 base pair (bp) cDNA insert of phage λ 42-4 (nucleotides 337–1,643; Fig. 1). Alignment of the cDNA sequence with the 42-mer probe sequence gave the proper reading frame of the cDNA and demonstrated that it did indeed encode TNF. Of the 14 amino acids (residues 104–117, Fig. 1) assigned to tryptic peptide TD-6 on the basis of preliminary protein sequence, 13 were correct; the only discrepancy was in the last amino acid (position 117) where the cDNA sequence encodes a proline residue rather than the predicted lysine. Despite this difference, the hybridization of the synthetic probe to the TNF cDNA clone was successful, as the 42-mer matched the cDNA sequence in 34 of the first 38 positions, including a stretch of 17 consecutive homologous nucleotides (nucleotides 711–727; Fig. 1).

The assignment of valine (residue 1, Fig. 1) as the first residue of mature TNF was based on NH₂-terminal protein sequence analysis of the intact molecule (Val-Arg-Ser-Ser-Ser- - -)¹⁶. There are 156 amino acids encoded after this valine before an in-phase termination codon occurs. The coding region of TNF is followed by 792 nucleotides of 3' untranslated sequence containing the hexanucleotide AATAAA (position 1,630–1,635) which precedes the site of polyadenylation in most eukaryotic mRNAs²⁷.

Additional confirmation that this sequence codes for TNF was obtained by determining the amino acid sequence of nine tryptic peptides of natural HL-60 TNF and several peptides generated by digestion with *S. aureus* V8 protease and chymotrypsin¹⁶. The M_r of 17,356 calculated for the mature TNF monomer from the cDNA sequence agrees closely with the value obtained for natural TNF by SDS-polyacrylamide gel electrophoresis and amino acid composition¹⁶. These results and

Table 2 Necrosis of MethA sarcoma *in vivo* by TNF

Treatment	Necrotic response			
	+++	++	+	-
	No. of mice			
PBS, i.l.	0	1	1	9
PBS, i.p.	0	0	1	4
PBS, i.m.	0	0	1	4
<i>E. coli</i> LPS, i.l.	0	0	1	9
HL-60 TNF, i.l.	5	1	1	0
rTNF, i.l.	7	5	0	0
rTNF, i.p.	2	1	2	0
rTNF, i.m.	2	2	1	0

(BALB/c×C57BL/6)F₁ female mice were injected intradermally with 5×10^5 BALB/c MethA sarcoma cells. Ten days later, the tumours (0.75 cm average diameter) were injected intralesionally (i.l., 1×10^5 U), intraperitoneally (i.p., 5×10^5 U) or intramuscularly (i.m., 5×10^5 U) with TNF in a total volume of 0.1 ml PBS. At 24 h after TNF treatment the tumours were excised, sectioned and scored for haemorrhagic necrosis by visual and histological examination as described previously¹. In the maximum response (+++) 50–75% of the tumour mass is markedly necrotic after 24 h; ++ denotes a moderate response, that is 25–50% haemorrhagic necrosis; +, a minimal response of <25% haemorrhagic necrosis; –, tumours showed no visible necrosis. Natural TNF was purified from HL-60 cultures as described elsewhere¹⁶. Recombinant TNF (rTNF) was purified from *E. coli* W3110/pTNFtrp to a purity of >95% and a specific activity of $\sim 10^8$ units mg^{-1} (T. Bringman, unpublished results).

the absence of any potential *N*-glycosylation sites in the deduced amino acid sequence suggest that TNF is not a glycoprotein. These data suggest also that TNF may occur naturally in multimeric form, as the *M_r* estimated previously for human TNF ranged from 34,000–140,000 (refs 6, 28). There are two cysteine residues (positions 69 and 101) in TNF which are probably involved in a single intramolecular disulphide bond¹⁶.

The cDNA clone λ 42-4 contains the entire coding region of mature TNF but lacks a complete signal peptide coding sequence and initiation codon. To obtain the missing sequence information, a specifically-primed cDNA library was prepared (see Fig. 1 legend) and screened with the ³²P-labelled λ 42-4 cDNA insert. A cDNA clone (λ 16-4) was identified which contained an insert extending 337 bp further 5' than the λ 42-4 insert (Fig. 1).

From an analysis of the TNF cDNA sequence, it seems that TNF is synthesized initially as part of a larger precursor (pre-TNF). Starting at the 5' end of the cDNA, 125 nucleotides of non-translated sequence are followed by a methionine codon and an open reading frame of 233 amino acids. This AUG is preceded by termination codons in all three frames, suggesting that it is the initiation codon. Furthermore, the sequence context of this AUG conforms closely to the CC₆CCAUGG proposed²⁹ as a consensus sequence for eukaryotic initiator sites.

The presequence of 76 residues is most probably involved in the secretion of TNF as it is not observed on the mature TNF polypeptide and contains an unusually long hydrophobic region of 26 amino acids (residues –46 to –21). Typically, signal peptides involved in protein secretion are only 20–30 amino acids long^{30,31}. However, a signal sequence for the Rous sarcoma virus envelope glycoprotein³² is atypically long (63 residues) and contains also many charged amino acids at its amino terminus, such as pre-TNF. It is interesting to note the presence of Arg-Arg and Lys-Lys dipeptides in the first 30 amino acids of the TNF pre-sequence, as pairs of basic amino acids often serve as cleavage sites for the release of physiologically-important peptides from precursor molecules^{33–36}.

We used the ³²P-labelled λ 42-4 cDNA insert to examine TNF gene structure and mRNA size. Results from Southern³⁷ hybridizations indicate that only a single gene for TNF is present in the human genome. Northern hybridization analysis³⁸ shows that a single mRNA species ~ 18 S in size is synthesized in PMA-induced HL-60 cultures and BCG/LPS-treated macrophages isolated from human PBLs. This provides additional evidence that the same cytotoxin is produced from both

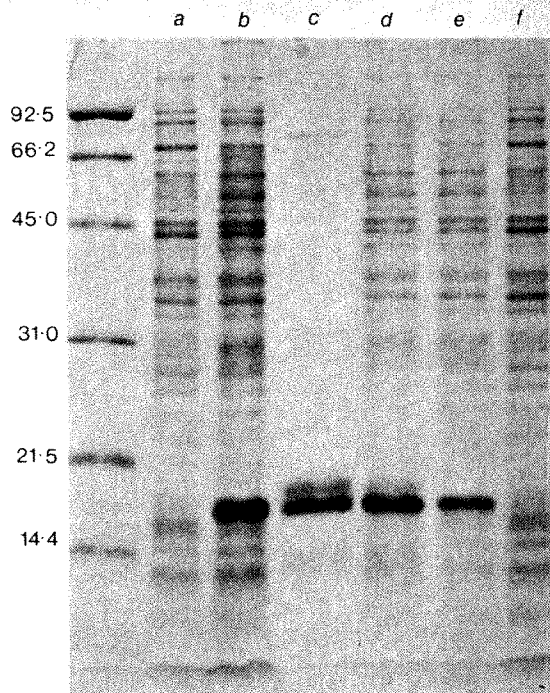


Fig. 3 SDS-polyacrylamide gel electrophoresis of human TNF synthesized in *E. coli*. *E. coli* K-12 strain W3110, transformed with pTNFtrp or pBR322, was grown in M9 medium containing $5 \mu\text{g ml}^{-1}$ tetracycline. Cells were collected, lysed in 2% SDS, 1% β -mercaptoethanol and precipitated with 10 volumes of cold acetone. Samples were electrophoresed on a 12.5% SDS-polyacrylamide slab gel using the buffer system of Maizels³² and the gel stained with Coomassie brilliant blue. The left lane contains protein *M_r* standards ($\times 10^{-3}$): phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,500). Lanes a, f, cell lysates of *E. coli* W3110/pBR322; lanes b, e, cell lysates of *E. coli* W3110/pTNFtrp; lane c, partially purified human TNF isolated from the HL60 cell line¹⁶; lane d, mixture of the *E. coli* W3110/pTNFtrp cell lysate and the HL60-derived, purified TNF.

cell sources and suggests that the TNF cDNA sequence shown in Fig. 1 represents a nearly full-length copy of TNF mRNA. No hybridization was detected to mRNA isolated from uninduced cultures (data not shown).

TNF synthesis in *E. coli*

Proof that the cDNA described here encodes TNF requires the demonstration that it can direct the synthesis of a gene product with the properties of authentic human TNF. To allow characterization of the protein encoded by the cloned cDNA, we engineered the TNF cDNA sequence for direct expression in *E. coli* (Fig. 2). In the resulting expression plasmid, pTNFtrp, the TNF DNA sequence is under the transcriptional control of a 300-bp DNA fragment of the *E. coli trp* operon containing the *trp* promoter, operator and Shine-Dalgarno sequence of the *trp* leader peptide.

Total extracts of *E. coli* K-12 strain W3110 transformed with pTNFtrp contained a prominent polypeptide with an apparent *M_r* 17,000 (Fig. 3, lanes b, e). This protein is not visible in cells transformed with pBR322 (lanes a, f), strong evidence that it represents the translational product of the TNF cDNA sequence. Furthermore, this protein co-migrates with authentic TNF (lane c) isolated from the HL-60 cell line (lane d), suggesting that no significant post-translational processing of TNF occurs in the HL-60 cell line. This is unlike lymphotoxin and γ -interferon, both of which occur naturally as heterogeneous glycoproteins as a consequence of N-terminal¹² and C-terminal³⁹ proteolysis, respectively.

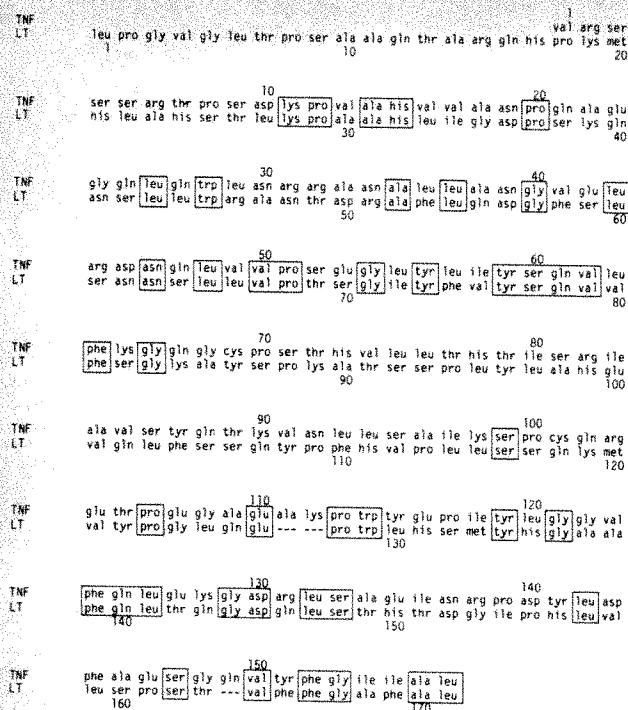


Fig. 4 Comparison of the amino acid sequence of human TNF with human lymphotoxin^{12,13}. The sequences have been aligned to give maximal homology by introducing two gaps (indicated by dashed lines) into the lymphotoxin sequence. Identical amino acids are boxed. The numbers above each row (1–157) and below each row (1–171) indicate the amino acids of mature TNF and lymphotoxin (LT), respectively.

Verification of the bacterial production of biologically-active TNF was obtained by assaying extracts of *E. coli* W3110/pTNFtrp for cytolytic activity in the murine L-929 fibroblast assay¹¹. Approximately 300,000 units of activity were detected per ml of culture at $A_{550}=1$, whereas no activity was observed in *E. coli* W3110/pBR322 controls. This corresponds to ~3 mg of active TNF per l ($A_{550}=1$) or about 300,000 molecules of active TNF per cell if a specific activity of 10^8 units mg^{-1} (ref. 16) is assumed. The activity was neutralized by antiserum prepared against partially purified PBL-derived TNF, but was not neutralized by preimmune serum or rabbit anti-human lymphotoxin antibodies (data not shown).

In vivo necrosis activity

TNF is generally defined as a cytotoxin released by BCG/LPS-treated macrophages which induces the haemorrhagic necrosis of methylcholanthrene-induced (MethA) sarcomas in BALB/c mice^{1,2}. Therefore, we examined recombinant human TNF purified from *E. coli* and natural human TNF from PMA-induced HL-60 cultures for *in vivo* tumour necrosis activity in the MethA assay¹. Both recombinant and natural TNF samples elicited significant necrotic responses, regardless of whether the TNF was injected intralesionally or systemically (Table 2). Minimal or no necrosis of the MethA sarcoma tumours was observed in mice injected with either phosphate-buffered saline (PBS) or 100 μg *E. coli* LPS. These results, taken with the antibody neutralization and Northern hybridization data, provide further evidence that the cytotoxin described here is human TNF.

Homology to lymphotoxin

The known *in vivo* and *in vitro* biological activities of TNF and lymphotoxin are very similar^{2,3,13}. TNF and lymphotoxin are now known to be antigenically distinct molecules³. It has thus become common to distinguish these two lymphokines on the basis of the cell populations responsible for their synthesis. We have compared the amino acid sequences of human TNF and

lymphotoxin to determine whether the similarities in their biological properties might be attributed to common structural features (Fig. 4). By introducing two gaps, the lymphotoxin sequence can be aligned with the TNF sequence so that distinct homologies are apparent; we find 44 of the 157 TNF residues (28%) are identical to corresponding lymphotoxin amino acids with many of the remaining differences between the two polypeptides resulting from conservative amino acid changes. The nucleotide homology over this coding region is 46% (data not shown). Two particularly conserved regions occur at amino acids 35–66 and 110–133 (TNF numbering) where 50% of the residues (28 of 56) are identical for TNF and lymphotoxin. The hydrophobic carboxy-termini of the two molecules are also significantly conserved. It is probable that the conserved regions are crucial to the shared cytotoxic activities of TNF and lymphotoxin, perhaps through interaction with a common receptor expressed on the surface of transformed cells. Support for this hypothesis is provided by the lack of cytotoxic activity in a truncated lymphotoxin polypeptide lacking its last 16 amino acids¹³.

Lymphotoxin has 18 more NH_2 -terminal amino acids than TNF (Fig. 4), suggesting that this region is not required for cytotoxic activity. In fact, a 148 residue lymphotoxin, consisting of amino acids 24–171 of mature lymphotoxin, and having similar cytotoxic effects on L-929 cells, has been isolated from the RPMI-1788 cell line^{11,12}. It is also interesting that amino acids 67–109 of TNF are unrelated to the corresponding region of lymphotoxin; only two of 43 residues are identical. This region includes all of the amino acids spanned by the Cys 69–Cys 101 disulphide bridge of TNF. One possible role for this non-conserved region could be to position correctly the two surrounding homologous regions in a conformation essential for cytotoxic activity. Such positioning, which could be achieved by a TNF disulphide bond, may require a very different sequence of amino acids in lymphotoxin, where no disulphide bond exists. These apparently unrelated regions of TNF and lymphotoxin might specify also as yet undiscovered differences in biological activities and/or target sites between the two molecules. The availability of efficient expression systems for TNF and lymphotoxin¹³, in combination with the techniques of site-directed mutagenesis⁴⁰, will make it possible to address questions of this type directly.

We thank Phil Hass for growing HL-60 cells; Dr Lloyd Svedersky and Tim Bringman for preparing TNF antiserum; Mark Vasser, Parkash Jhurani and Peter Ng for deoxyoligonucleotide synthesis; Irene Figari and Refaai Shalaby for assistance with the tumour necrosis assays; Roxanne Chang and the Genentech Bioassay Group for performing *in vitro* TNF assays; and Dr Richard Harkins for helpful suggestions. G.E.N. dedicates this work to the late Jack L. Levenson.

Received 20 August; accepted 9 November 1984.

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Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing

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A novel mammalian neuropeptide, the tachykinin substance K, is specified by a discrete genomic segment. Alternative RNA splicing generates two distinct mRNAs encoding the neuropeptide substance P alone or with substance K from a single preprotachykinin gene. Relative amounts of the mRNAs vary in different tissues, suggesting that the substance K-encoding sequence is regulated in a tissue-specific manner.

SUBSTANCE P is one of the best characterized neuropeptides in mammalian tissues; several lines of evidence suggest that it acts as a neurotransmitter or neuromodulator in primary sensory neurones¹. Substance P belongs to a family of related peptides, the tachykinins, and is thought to be the only member of this family present in mammalian tissues². Recently, we elucidated the entire primary structures of two types of bovine brain substance P precursors (α - and β -preprotachykinins) by determining their cloned cDNA sequences³. β -Preprotachykinin (β -PPT) contains not only the substance P sequence but also a novel tachykinin sequence designated substance K, whereas α -preprotachykinin (α -PPT) lacks the latter sequence, containing only substance P. The decapeptide substance K has been found independently as neurokinin α , a gut-contracting peptide in porcine spinal cord⁴. The chemically synthesized substance K peptide possesses biological activities characteristic of the tachykinin family, but is considerably more potent than substance P in some pharmacological tests^{5,6}. Substance K thus represents a second type of mammalian tachykinin which may have a physiological role different from that of substance P in mammalian organisms.

The two PPT mRNAs exhibit an interesting structural relationship. They have complete identity in their 5' and 3' sequences and differ only in the insertion/deletion of the sequence coding for the substance K region³. This characteristic structural relationship poses intriguing questions about the gene organization for these two mRNAs and the regulation for the generation of the two biologically different mammalian tachykinins. Our present investigations thus concern the structural organization of the PPT gene and the distribution and regulation of the two PPT mRNAs in the nervous system and peripheral tissues. We report here that the sequence specifying the substance K region is encoded by a discrete genomic segment, and that both α - and β -PPT mRNAs arise from a single gene by alternative RNA splicing events. We also present evidence indicating the tissue-specific regulation of the PPT gene for the differential generation of the two PPT mRNAs.

PPT gene organization

Genomic clones containing the bovine preprotachykinin gene were isolated from a bovine genomic library by hybridization

in situ with a bovine β -PPT cDNA probe, and all the isolated genomic DNA fragments were arranged into an approximately 36 kilobase-pair (kbp) length of a continuous genomic DNA (Fig. 1a; see Fig. 1 legend for experimental details of cloning). Nucleotide sequence analysis was performed on DNA fragments containing exons and their surrounding regions (Fig. 1b-f). Comparison of the genomic DNA sequence with the cDNA sequence enabled us to construct a structural organization of the bovine PPT gene (Fig. 1g). Intron A (403 base pairs, bp) is inserted within the segment encoding the 5'-untranslated region of the mRNA, 9-10 bp upstream from the translational initiation site. Introns B (~1.0 kbp), C (~450 bp), D (~460 bp), E (~1.4 kbp) and F (~3.6 kbp) all interrupt the protein-encoding region of the gene. The sequences at the exon-intron boundaries are consistent with the splice junction sequences observed for other genes⁷. Exons 2-7 consist of 132, 97, 45, 24, 54 and 596 bp, each encoding the protein sequence corresponding to the signal peptide, substance P, two spacer sequences, substance K, and the C-terminal sequence, respectively. It is remarkable that exon 6 precisely specifies the substance K region missing in α -PPT. Because blot-hybridization analysis of total cellular DNAs (data not shown) as well as the genomic cloning described above showed that no more than one PPT gene is present in the bovine genome, we conclude that both α - and β -PPT mRNAs are produced from a single gene as a consequence of alternative RNA splicing events.

The 5' termini of the PPT mRNAs were identified by S₁ nuclease mapping and primer extension analyses (Fig. 2). Both analyses revealed a length heterogeneity at the 5' end of the PPT transcripts. The major 5' termini of the PPT mRNAs mapped at 106-108, 110 and 111 bp upstream from the 3' end of exon 1 (Fig. 1g). Several minor mRNA species starting further upstream were also observed and these 5' termini mapped at roughly 132, 133, 137 and 146 bp upstream from the 3' end of exon 1. In support of these assignments, we found that three of the four cDNA clones isolated previously³ (clones pSP301, 302 and 306) contained the extreme 5' sequences corresponding to the major 5' ends, while the remaining one (clone pSP307) extended its 5'-terminus up to one of the minor 5' ends. Based on the assignments of the 5' termini of the PPT mRNAs, we conclude that the bovine PPT gene is ~8.4 kbp long.

Table 1 Relative levels of PPT mRNAs and relative amounts of α - and β -PPT mRNAs in bovine nervous system and peripheral tissues

Region or tissue assayed	β -PPT mRNA/ PPT mRNAs α -PPT mRNA	
	PPT mRNAs	α -PPT mRNA
Trigeminal ganglion	100	0.65 (0.49)
Caudate nucleus	70	0.29 (0.31)
Putamen	42	0.29 (0.33)
Olfactory bulb	32	0.83 (0.48)
Midbrain	20	0.28 (0.28)
Hypothalamus	9	0.31 (0.33)
Amygdala	6	0.38 (0.30)
Medulla oblongata	5	0.26 (0.24)
Thalamus	2	ND (0.30)
Pons	<1	ND (0.28)
Thyroid	2	>30 (>30)
Duodenum	2	9 (5)
Small intestine	1	7 (4)

The relative levels of PPT mRNAs in the brain region and peripheral tissues (left-hand column) are expressed as per cent of that in the trigeminal ganglion. Besides the above tissues, a little of the PPT mRNAs was identified in the adrenal medulla and none of the PPT mRNAs were detected in the bovine kidney, liver, urinary bladder or submaxillary gland. The ratios of β -PPT mRNA to α -PPT mRNA measured with 3'-end-labelled cDNA probe are presented in the right-hand column; values in parentheses indicate results obtained with 5'-end-labelled cDNA probe. ND, not determined because of faint bands. The relative levels of PPT mRNAs and the relative amounts of α - and β -PPT mRNAs were quantitated by densitometric scan analysis of autoradiographs of RNA blot-hybridization and S_1 nuclease protection analyses respectively. The RNA blot-hybridization analysis was performed as described previously²⁸ using total RNA (40 μ g) extracted²⁹ from various bovine brain regions and peripheral tissues; the hybridization probe used ($\sim 1.0 \times 10^6$ c.p.m. μ g⁻¹, 10 ng ml⁻¹) was the 1,066-bp *Rsa*I fragment derived from pSP307 (ref. 3) and labelled by nick-translation³⁰. The S_1 nuclease protection assays were carried out as described for Fig. 4. In both RNA blot-hybridization and S_1 nuclease protection analyses, the densitometric scan analysis was adjusted for the time of exposure such that each band analysed was within the linear portion of the exposure. The values given are based on multiple independent experiments.

Distribution of PPT mRNAs

To investigate the tissue distribution of bovine PPT mRNAs and their regional distribution in the brain, RNAs isolated from various tissues and brain regions were analysed by blot-hybridization techniques. Because α - and β -PPT mRNAs did not separate on agarose gel electrophoresis (data not shown), the sum of the PPT mRNA levels were quantified by scanning densitometry of autoradiographic exposures of the blot-hybridization analysis (Table 1, left column).

Previous studies combining surgical manipulations with immunohistochemistry identified various substance P-projecting neuronal pathways in the central and peripheral nervous system⁸. Figure 3 illustrates schematically the relationship between the major substance P-projecting pathways in the brain and the regional distribution of the PPT mRNAs determined in the present study. We find good correlation between the regions or nuclei that exhibit high levels of PPT mRNAs and those projecting the substance P-containing fibres. Because these regions express α -PPT mRNA over β -PPT mRNA, the substance P immunoreactivity identified in these regions may reflect mainly the peptide derived from α -PPT. It is thus possible that some of the substance K peptide derived from β -PPT may be transported to different nerve terminals as a neurotransmitter or neuromodulator for a different neuronal communication.

Expression of the PPT gene

To examine possible differences in the expression of the two mRNAs in the central nervous system (CNS) and in peripheral tissues, we next investigated the relative amounts of bovine α - and β -PPT mRNAs in various tissues and in various brain regions by S_1 nuclease protection analysis. Two cDNA probes were used for this analysis (Fig. 4c, d), and the results obtained

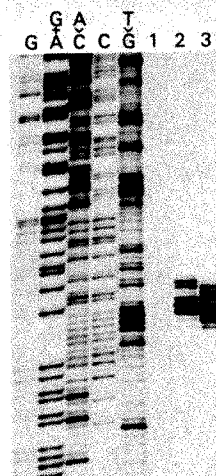


Fig. 2 Identification of the 5' termini of bovine PPT mRNAs by S_1 mapping and primer extension analyses. An autoradiograph shows the chemically degraded products used as a chain length marker (five left lanes), the S_1 digestion products using yeast tRNA (lane 1) and striatal poly(A) RNA (lane 2) and the primer-extended cDNAs using striatal poly(A) RNA (lane 3).

Methods: Poly(A) RNA was isolated from bovine striata³. The *Nci*I fragment containing most of the exon 1 sequence and its 5'-flanking sequence (Fig. 1b) was isolated from the 2.6-kbp *Bam*HI fragment containing exons 1 and 2, labelled with ³²P at the 5' ends and cleaved by *Hae*III or *Fnu*4HI. The 332-bp *Nci*I-*Hae*III fragment (15 fmol, 1.0×10^5 c.p.m.) and the 38-bp *Nci*I-*Fnu*4HI fragment (15 fmol, 1.0×10^5 c.p.m.) were used for S_1 mapping³⁵ and primer extension analyses³⁶, respectively; both DNAs were labelled at the same *Nci*I site present 17 nucleotides upstream from the 3' end of exon 1 (Fig. 1g). The S_1 digestion products and the primer-extended cDNAs were electrophoresed parallel to chemically degraded products of the 332-bp *Nci*I-*Hae*III fragment on 8.3 M urea/8% polyacrylamide gel.

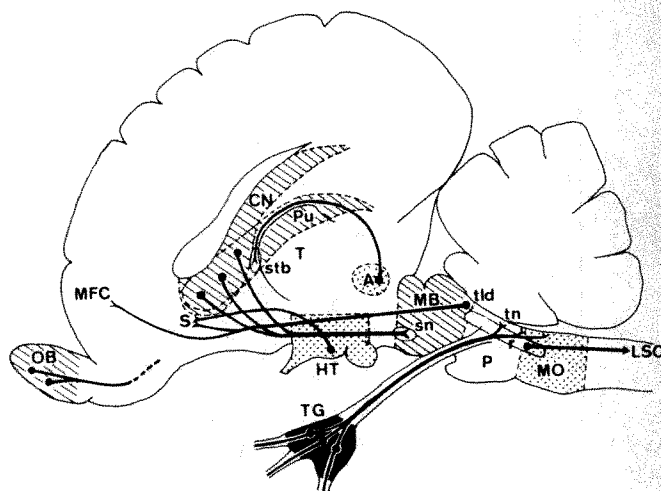
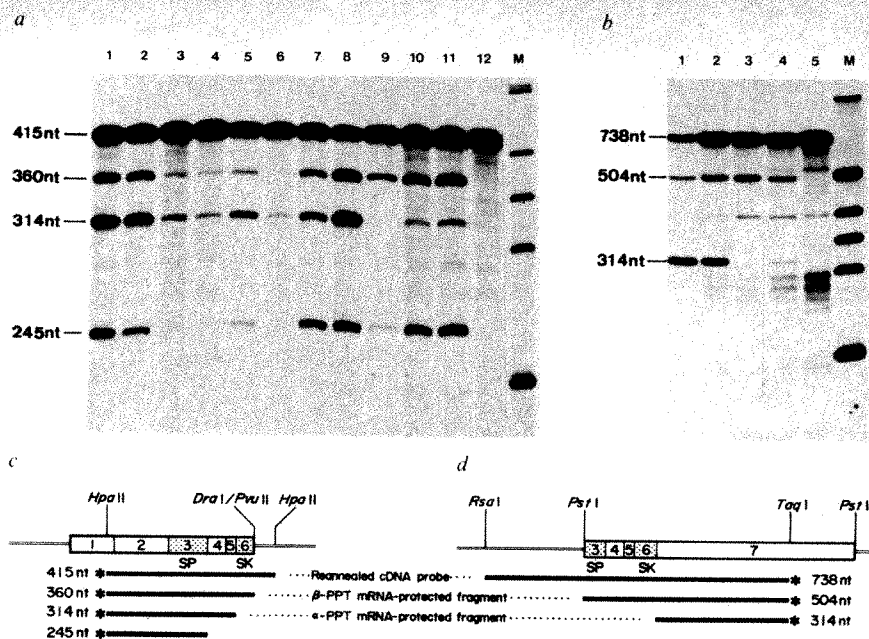


Fig. 3 The relationship between the regional distribution of PPT mRNAs and the major substance P-projecting pathways. According to the relative levels of PPT mRNAs in various brain regions presented in Table 1 (left column), the regions which exhibit high levels of PPT mRNAs are classified into three groups: black area, 100 (the trigeminal ganglion); shaded area, 20-70; dotted area, 5-9. The major substance P-projecting pathways are based on data reviewed in ref. 8: TG, trigeminal ganglion; OB, olfactory bulb; MFC, medial frontal cortex; CN, caudate nucleus; Pu, putamen; T, thalamus; A, amygdala; HT, hypothalamus; MB, midbrain; P, pons; MO, medulla oblongata; stb, bed nucleus of stria terminalis; S, lateral septal area; sn, substantia nigra; tld, nucleus laterodorsalis tegmenti; tn, spinal nucleus of trigeminal nerve; r, raphe nuclei; LSC, lumbar spinal cord.

Fig. 4 S_1 nuclease protection analysis of PPT mRNAs. *a, b*, Autoradiographs of *c, d*; *c, d*, diagrams of the S_1 nuclease-resistant DNA fragments (thick lines) in the protection assays using 3'- and 5'-end-labelled cDNA probes. *a*, RNAs analysed as follows: lane 1, caudate nucleus; lane 2, putamen; lane 3, hypothalamus; lane 4, amygdala; lane 5, mid-brain; lane 6, medulla oblongata; lane 7, olfactory bulb; lane 8, trigeminal ganglion; lane 9, thyroid; lane 10, duodenum; lane 11, small intestine; lane 12, liver (control). M, ^{32} P-labelled markers of *Hind*III-digested pBR322 DNA. *b*, The following RNAs were used: lane 1, hypothalamus; lane 2, trigeminal ganglion; lane 3, thyroid; lane 4, small intestine; lane 5, liver (control). M, markers. *c, d*, Open boxes and double lines at the top indicate the cDNA sequences and their flanking vector sequences, respectively; the numbered boxes indicate the corresponding exon sequences, and the dotted boxes encode the substance P (SP) substance K (SK) sequence. The asterisks denote the ^{32} P-labelled sites. nt, Nucleotides.

Methods: *a*, The ~750-bp *Hind*III-*Dra*I fragment containing a portion of the β -PPT cDNA sequence and its flanking vector sequence was isolated from clone pSP302 (ref. 3) and subcloned into pBR322 at the *Hind*III and *Pvu*II sites (clone pPPT $_{\beta 2}$). pPPT $_{\beta 2}$ DNA was digested with *Hpa*II and was ^{32} P-labelled at the 3' ends with [α - ^{32} P] dCTP. The 415-bp *Hpa*II fragment was isolated and used as a probe. This probe contained the nucleotide sequence complementary to 360 nucleotides of β -PPT mRNA and 55 nucleotides of the vector sequence. The probe (6.3 fmol, 4.0×10^4 c.p.m.) was denatured, hybridized to total RNA (40 μ g, lanes 1-8, 12) or poly(A) RNA (40 μ g, lanes 9-11) and digested with S_1 nuclease (10 U per μ g RNA). *b*, The 661-bp *Pst*I fragment containing a portion of the β -PPT cDNA sequence was isolated from clone pSP307 (ref. 3) and subcloned into pBR322 (clone pPPT $_{\beta 1}$). pPPT $_{\beta 1}$ DNA was digested with *Taq*I, ^{32} P-labelled at the 5' ends and cleaved by *Rsa*I. The 738-bp *Taq*I-*Rsa*I fragment used as a probe contained the nucleotide sequence complementary to 504 nucleotides of β -PPT mRNA and 234 nucleotides of the vector sequence. The probe (17 fmol, 1×10^5 c.p.m.) was denatured, hybridized to poly(A) RNA (10 μ g) and digested with S_1 nuclease (10 U per μ g RNA). The S_1 digestion products were electrophoresed parallel to the markers on 8.3 M urea/8% polyacrylamide gel. Exposure time for lanes 3-5 is three times longer than that for lanes 1-2 in *b*.



with use of the 3'- and 5'-end-labelled cDNA probes are presented in Fig. 4*a* and *b*, respectively. The analysis with the former cDNA probe gave rise to three RNA-protected bands with estimated sizes of 360, 314 and 245 nucleotides. The former two sizes corresponded to those representing the cDNA fragments protected by β - and α -PPT mRNAs, respectively. The appearance of the 245-nucleotide fragment on this S_1 nuclease analysis, however, was unexpected. The size of this fragment corresponded to the distance between the 3'-end-labelled *Hpa*II site and the 3' end of exon 3. This coincidence suggests that splicing at the 3' end of exon 3 may occur in the expression of the PPT gene, and that this different mode of RNA splicing may generate a third PPT mRNA. The analysis using the 5'-end-labelled cDNA probe, however, revealed two and not three RNA-protected bands, and the sizes of these two bands (504 and 314 nucleotides) corresponded to those representing the cDNA fragments protected by β - and α -PPT mRNAs, respectively. If the third PPT mRNA indeed exists in various tissues, the absence of the corresponding protection band on the second S_1 nuclease analysis may imply that there is an unidentified acceptor splice site for the formation of the third PPT mRNA. Another possible explanation is that the S_1 nuclease-resistant fragment protected by the third PPT mRNA by chance overlaps with the ~380 nucleotide band observed in the control lane. If so, the size of this fragment suggests that the acceptor splice site for the formation of the third PPT mRNA is located at the 5' end of exon 5. However, further characterization of the possible third PPT mRNA remains to be determined.

Densitometric scannings of the autoradiographs of the S_1 nuclease protection analyses enabled us to quantitate the relative amounts of α - and β -PPT mRNAs in the bovine nervous system and peripheral tissues irrespectively of the third PPT mRNA (Table 1, right column). There is a good agreement between the values obtained with the two different probes. Table 1 shows clearly that there are marked variations in the relative amounts of α - and β -PPT mRNAs among the various tissues tested. The level of α -PPT mRNA is two to three times higher than that of

β -PPT mRNA in the nervous system. In peripheral tissues, this relation of the two mRNAs is completely reversed and the level of β -PPT mRNA is considerably higher than that of α -PPT mRNA. This is particularly remarkable in the thyroid gland where virtually no α -PPT mRNA was detected (Fig. 4*a*, lane 9; *b*, lane 3). The observed tissue-specific variations in the relative amounts of the two mRNAs are independent of the levels of the PPT mRNAs (Table 1). The CNS exhibits an almost constant ratio of the two mRNAs in spite of the marked differences in the levels of the PPT mRNAs, with the exception of the olfactory bulb. We therefore conclude that the expression of the two PPT mRNAs is regulated in a tissue-specific manner via the specific inclusion or exclusion of a peptide-coding sequence by means of different RNA splicing events.

Figure 5 schematically illustrates the expression of the two PPT mRNAs. α -PPT mRNA is mainly synthesized in the nervous system and codes for the substance P-containing precursor, whereas β -PPT mRNA is produced in both the nervous system and some peripheral tissues and codes for the substance P- and substance K-containing precursor. The two mammalian tachykinins substance P and substance K exhibit markedly different biological activities on a wide range of mammalian tissues^{5,6}; such differences can be ascribed to the existence of at least two types of the tachykinin receptors, termed SP-P and SP-E receptors^{5,9}. The two receptors are supposed to have different affinities for substance P and substance K and to be distributed differently in various mammalian tissues. Thus, through the selectivity of the two tachykinin receptors, the differential expression of the two mRNAs from the single PPT gene would ultimately produce different physiological responses in the CNS as well as in peripheral tissues.

Conclusions

Examples of alternative RNA splicing for the generation of several mRNAs from a single gene have been reported for some viral systems^{10,11} and cellular genes¹²⁻²¹. Apart from viral genes, examples of sequence differences between mRNAs from a single

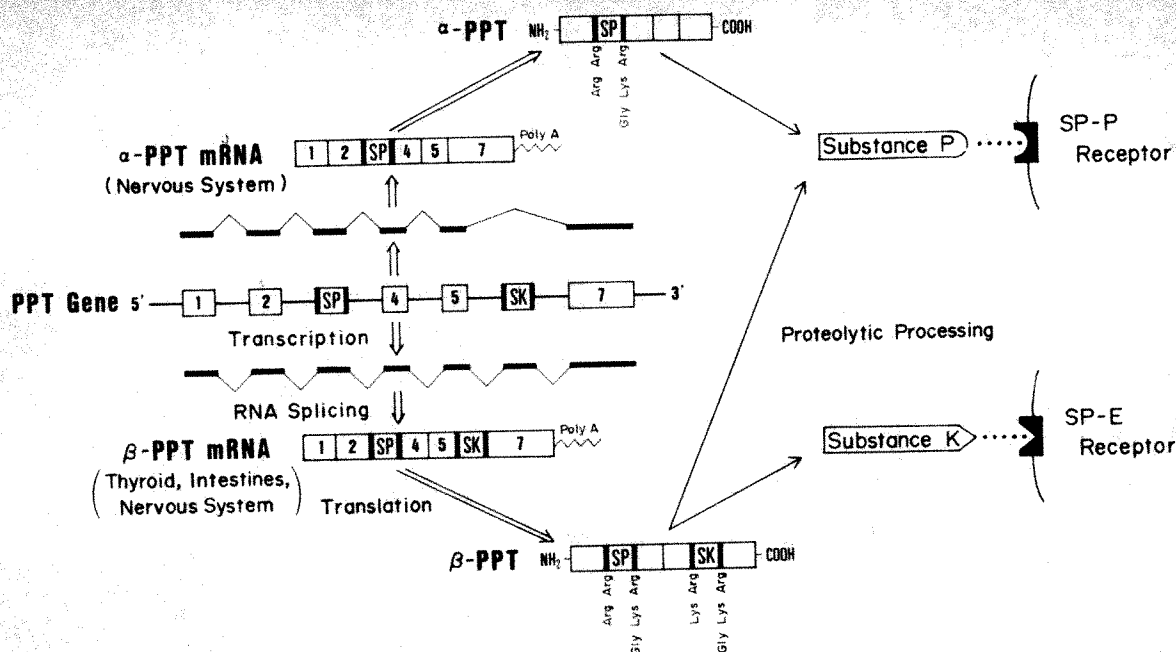


Fig. 5 Schematic representation of alternative RNA splicing pathways in the expression of the PPT gene. In the structure of the PPT gene, numbered boxes and those indicated as SP and SK stand for the corresponding exons and the exons encoding substance P (exon 3) and substance K (exon 6), respectively. Tissues in which β -PPT mRNA is predominantly expressed or both α - and β -PPT mRNAs are expressed are shown in parentheses. Amino acids under the protein structures indicate those involved in the proteolytic processing and the C-terminal amidation for the formation of substance P and substance K.

gene predominantly concern differences in the 5' and 3' ends of the mRNAs¹²⁻¹⁷. In these cases, the generation of different mRNAs could result from the use of alternative promoters or polyadenylation sites. In contrast, the PPT gene represents a novel example in which differential splicing events seem to be independent of alternative promoters or polyadenylations. The inclusion or exclusion of a separated exon sequence by alternative splicing, as described here, has been reported for the *Drosophila* myosin gene²⁰ and the murine α A-crystallin gene²¹. In the previously reported cases, however, it has not been clear whether alternative splicing events serve any functions. The PPT gene thus provides an intriguing system in which the inclusion or exclusion of a whole exon sequence by alternative RNA splicing events leads to the production of functionally different polypeptides.

The structure of the PPT gene and its RNA products strongly suggest that the true regulation of splicing choice takes place in the PPT primary transcript and that this molecular mechanism is responsible for the observed tissue-specific alteration in the expression of the two PPT mRNAs. Such splicing regulation could arise from changes in enzyme specificity, small nuclear RNAs and other factors affecting the splicing process. The change of the chromatin structure or the modification of the gene structure may also affect the accessibility of the splicing machinery to a splice site. Another mechanism could involve the transcription initiation at different sites affecting a splice site of the PPT primary transcripts. This possibility seems unlikely because we identified the same multiple transcription initiation sites for both α - and β -PPT mRNAs on S_1 nuclease mapping analysis (data not shown). A further possibility is that although all the nucleotide sequences of the exon-intron junctions of the PPT gene agree with the consensus sequence found at the exon-intron boundaries of other genes⁷ (Fig. 1g), a small rearrangement of the gene structure or nucleotide changes in the gene may occur and change a splice site of the primary transcript.

The observed tissue-specific variations in the relative amounts of the two PPT mRNAs raise interesting questions about what types of cells express PPT mRNAs as well as whether there is cell-type-specific regulation of their expression. It was shown

previously that enterochromaffin cells are the main substance P-immunoreactive cells in the intestines²². Furthermore, the medullary carcinoma of the thyroid was found to contain large amounts of substance P²³. Thus, enterochromaffin cells and thyroid C cells may involve the predominant expression of β -PPT mRNA in the intestines and thyroid. Similarly, a particular neurone may express either α - or β -PPT mRNA throughout its lifetime, implying the presence of separate groups of neurones containing substance P alone or both substance P and substance K. Alternatively, a given neurone may be able to switch its pattern of PPT mRNA production during development or in response to external stimuli. These questions are important for further understanding of the physiological role of the mammalian tachykinins.

Specific neurones or sets of neurones may use a variety of peptides to communicate with one another and to express an invariant programme during development. Such informational ability may have been expanded during evolution by the increase of the diversity of neuropeptides in several ways. These include gene duplication^{24,25}, repetition of multiple neuropeptides within a single precursor²⁵⁻²⁸ and alternative RNA processing in combination with different polyadenylations and RNA splicing¹⁵. We have presented here another novel example in which the diversity of neuropeptides can be increased by the specific inclusion and exclusion of the internally separated exon sequence encoding the neuropeptide. The characteristic feature of this system is that the regulation of the inclusion/exclusion of the peptide-coding sequence would ultimately change the ratio of the combination of the biologically different peptides. In addition, this system can simultaneously provide a means for the coordinate synthesis of the different peptides under the control of a single promoter, once the peptides are encoded in a single transcription unit. The PPT gene thus represents an interesting model system not only for exploring mechanisms responsible for the tissue-specific regulation in the expression of the eukaryotic gene but also for understanding the molecular basis for the generation of the diversity of neuropeptides in the neuroendocrine system.

This investigation was supported in part by research grants from the Ministry of Education, Science and Culture of Japan.

Received 24 June; accepted 31 August 1984.

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LETTERS TO NATURE

Nuclearites—a novel form of cosmic radiation

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E. Witten (personal communication) has raised the intriguing possibility that nuclear matter consisting of aggregates of up, down and strange quarks in roughly equal proportions may be less massive than ordinary nuclear matter of the same quark number consisting of protons and neutrons (triplets of non-strange quarks). These nuggets of strange quark matter may be stable for almost any baryon number (A), including values intermediate between those of ordinary nuclei ($A \leq 263$) and neutron stars ($A \sim 10^{57}$). We use the term 'nuclearite' to describe such strange quark nuggets in collision with Earth and suggest experiments to detect these encounters.

Ordinary nuclear matter has an energy density of ~ 938 MeV per nucleon. Ordinary quark matter—a purely hypothetical form of matter in which the constituent up and down quarks and gluons are not confined within individual nucleons—evidently has a higher energy density, for otherwise nuclei would decay into quark matter. Strange quark matter (consisting of equal numbers of up, down and strange quarks) differs energetically from ordinary quark matter in two ways: strange quarks are considerably heavier than ordinary quarks, which is why strange baryons are some 200 MeV heavier than nucleons. On the other hand, the Pauli exclusion principle acts so as to make strange quark matter more stable than ordinary quark matter. In a degenerate gas of massless quarks at zero temperature, the energy per quark depends on the number of quark species, and in a three-flavour system (strange quark matter) it is estimated by Witten to be $\sim 90\%$ of what it is in a two-flavour system. This additional (negative) symmetry energy may more than compensate the positive energy penalty associated with the strange quark mass.

Farhi and Jaffe¹ have performed semiquantitative calculations on the basis of the Massachusetts Institute of Technology bag model. They find that single nuggets of strange quark matter are stable for a range of strange quark masses and strong quantum chromodynamic (QCD) coupling constants in accordance with phenomenological estimates of these parameters extracted from hadron spectroscopy. In short, they are unable to decide definitely whether strange quark matter is stable or not. We may not conclude from the stability of nuclei that a stabler form of nuclear matter does not exist. The transition of, say, ^{56}Fe into strange quark matter containing 168 up, down

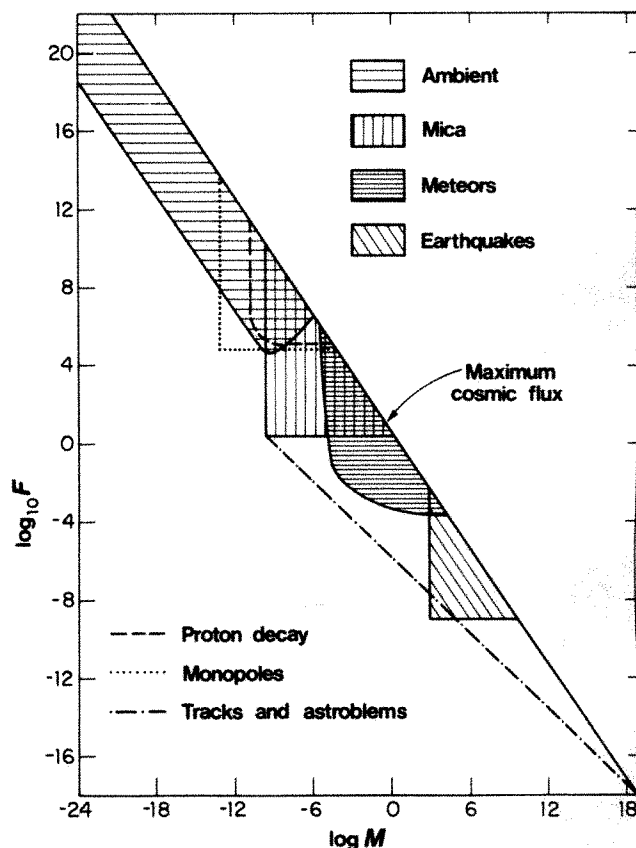


Fig. 1 The maximum possible cosmic flux of nuclearites F ($\text{km}^{-2} \text{yr}^{-1} (2\pi \text{sr})^{-1}$) as a function of mass. Also shown are regions that would be excluded by the several experiments discussed in the text. (Of these, only the 'mica' limit corresponds to an experiment actually performed.)

and strange quarks, in equal proportions, is a 56th-order weak interaction. Even if strange quark matter were stable, the lifetime of ordinary matter would be essentially infinite on cosmological time scales.

Witten offers a tentative view of the early Universe in which a large fraction of the mass and baryon number of the Universe has survived in the form of nuggets of strange quark matter (nuclearites) with radii R , between 0.1 and 10 cm, appearing shortly after the Big Bang in the QCD transition from a quasi-free quark plasma to a cooler state where quarks are confined. Strange quark matter is nonluminous (not the stuff of ordinary stars) and did not participate in primordial nucleosynthesis. Primordial nuclearites may constitute the dark missing mass of the Universe and of the Milky Way.

There may be more recent sources. What we regard as neutron stars may in fact consist of strange quark matter². Collisions

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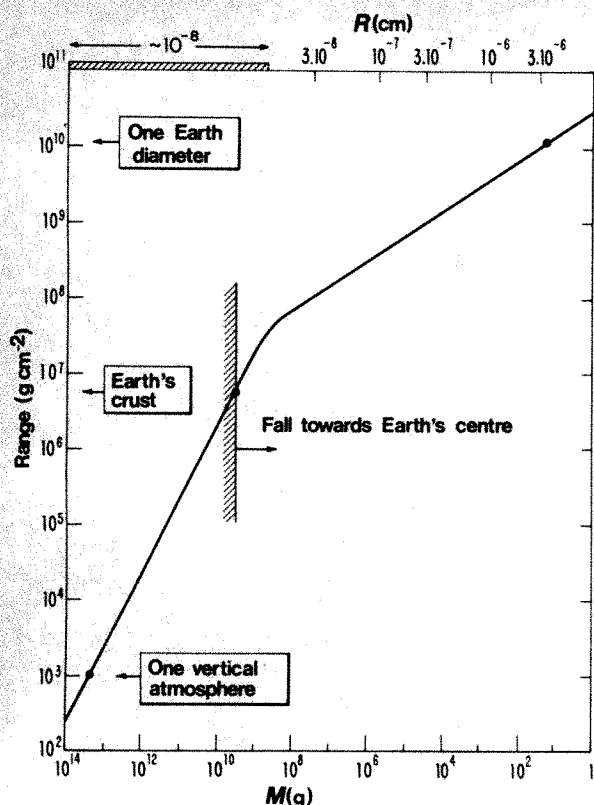


Fig. 2 The range of nuclearites as a function of their mass, as predicted by equation (5).

involving these stars, or supernovae themselves, may liberate pieces of strange quark matter with a spectrum of sizes that is difficult to guess. Nuclearites, or other unsuspected states of nuclear matter, may exist in the Galaxy as remnants of the Big Bang or as debris of astrophysical catastrophes.

Galaxies, our own included, contain significant non-luminous mass², possibly in the form of nuclearites. The local density of dark matter is estimated to be $\sim 10^{-24} \text{ g cm}^{-3}$. With chaotic velocities $\sim 250 \text{ km s}^{-1}$ characteristic of the Sun's galactic rotation, the local flux of dark matter (an upper bound to the nuclearite mass flux) is $\sim 2.5 \times 10^{-17} \text{ g cm}^{-2} \text{ s}^{-1}$ and the annual Earth infall is $\sim 10^9 \text{ g}$. We assume that the entire flux consists only of nuclearites so as to set minimum useful sensitivities on experiments designed to discover cosmic nuclearites. If the mean mass of a cosmic nuclearite is M , the maximal cosmic flux is given by

$$F = 7.8(1 \text{ g}/M) \text{ nuclearites km}^{-2} \text{ yr}^{-1} (2\pi \text{ sr})^{-1} \quad (1)$$

Equation (1) is plotted in Fig. 1 along with regions excludable by experiments we shall consider.

The density of strange quark matter is estimated² to be $\rho_N = 3.6 \times 10^{14} \text{ g cm}^{-3}$, somewhat larger than that of atomic nuclei or neutron stars. Because strange quarks are heavier than the others and so disfavoured, the net-quark charge is positive and is compensated by electrons (negative charge). Nuclearites, small compared with the Bohr radius, resemble superheavy nuclear atoms with $A < 10^{15}$ and Z well beyond any published in a periodic table. The electron distribution of larger nuclearites extends beyond the nuclear boundary by at least an electron-Compton wavelength. Nuclearites with galactic velocities are protected by their electron 'atmosphere' and coulomb repulsion from direct nuclear interactions with the atoms they may hit; the same is true of nuclearites that have come to rest in matter.

The principal energy-loss mechanism for a nuclearite passing through matter is that of atomic collisions. As it traverses the medium, it displaces all the matter in its path by elastic or quasi-elastic collisions with the ambient atoms. For a massive

nuclearite, as for meteorites⁴, the rate of energy loss is

$$\frac{dE}{dx} = -A\rho v^2 \quad (2)$$

where A is the effective cross-sectional area of the nuclearite, v is its velocity, and ρ is the density of the medium. Thus, v decreases exponentially with distance L according to

$$v(L) = v(0) \exp \left\{ -\frac{A}{M} \int_0^L \rho dx \right\} \quad (3)$$

where M is the mass of the nuclearite. In the case of a nuclearite larger than an atom ($R_0 \geq 1 \text{ \AA}$), its cross-sectional area is simply πR_0^2 . On the other hand, the effective area of a smaller nuclearite is controlled by its electronic atmosphere which is never smaller than $\sim 1 \text{ \AA}$. Thus, we take

$$A = \begin{cases} \pi(3M/4\pi\rho_N)^{2/3} & M \geq 1.5 \text{ ng}, \\ \pi \times 10^{-16} \text{ cm}^2 & M < 1.5 \text{ ng}. \end{cases} \quad (4)$$

Equation (2) breaks down at low velocity when the retarding force does not vanish but becomes equal (in a solid) to the force by which the confining material resists interpenetration. The integrity of rock persists up to pressures of 1,000 atm, corresponding to a structural energy density $\epsilon \approx 10^9 \text{ erg cm}^{-3}$, or $\approx 0.1 \text{ eV}$ per molecular bond. For subsonic velocities, $v(L) < v_c = \sqrt{\epsilon/\rho}$, the right hand side of equation (2) must be replaced by the constant force $-\epsilon A$, and from this point the nuclearite is rather quickly brought to rest.

From equation (2), and the subsequent discussion, we compute the range of a nuclearite as a function of its mass

$$\begin{aligned} \int_0^L \rho dx &= (M/A) \ln(v(0)/v_c) \\ &= \begin{cases} 3 \times 10^7 [M/1 \text{ ng}]^{1/3} \text{ g cm}^{-2} & M \geq 1.5 \text{ ng} \\ 2.3 \times 10^7 [M/1 \text{ ng}] \text{ g cm}^{-2} & M < 1.5 \text{ ng} \end{cases} \end{aligned} \quad (5)$$

From Fig. 2, we see that nuclearites heavier than $4 \times 10^{-14} \text{ g}$ penetrate the atmosphere while maintaining cosmic velocities and those heavier than 0.1 g pass freely through an Earth diameter whose column density (see, for example, ref. 5) is $1.1 \times 10^{10} \text{ g cm}^{-2}$.

Cosmic nuclearites of low-enough mass will come to rest in the Earth's crust and accumulate therein. The effect of gravity cannot be neglected: if the Earth's pull, Mg , exceeds the maximum static retarding force, ϵA , a nuclearite cannot come to rest, but will fall through the Earth. Nuclearites lighter than 0.3 ng will stop in the crust and can have developed an observable concentration. The maximal cosmic nuclearite flux given by equation (1), over the course of the Earth's 4.6-Gyr history will have produced a crustal concentration of 10^{-7} by mass. Searches for these ambient nuclearites require judicious choice of materials (deep sea sediments, excreta, ...), methods of concentration (distillation, centrifugation) and detection techniques (proton X-ray activation, mass spectroscopy, ...). The masses of accumulated nuclearites can range from those of atomic nuclei to the upper limit of 0.3 ng or $A \sim 2 \times 10^{14}$. A complete search probably requires the use of a variety of experimental approaches. Figure 1 shows the excluded region that would result from a negative search for crustal nuclearites to a concentration of 10^{-13} , a feasible experiment not yet performed.

Cosmic nuclearites, in traversing a transparent medium like water or air will deposit some of their energy in the form of visible light. The fraction of dissipated energy appearing as light is called the luminous efficiency η . A lower bound on η can be deduced from simple thermodynamical arguments in which light is emitted as black-body radiation from an expanding cylindrical thermal shock wave. The effective temperature of the shock depends on its radius, R , according to

$$T(t) = \frac{mv^2}{n} (R_0/R(t))^2 \quad (6)$$

where m is molecular mass of the medium and n is the effective

number of submolecular species that are excited. For this calculation, we use natural units where $\hbar = c = k = 1$. The rate of expansion of the shock is given by

$$\dot{R}(t) = \sqrt{\frac{2Tn}{m}} \quad (7)$$

characteristic of molecular velocities. From equations (6) and (7), we deduce

$$\begin{aligned} R^2(t) &= (8)^{1/2} vt R_0 \\ T(t) &= (8)^{-1/2} mv R_0 / nt \end{aligned} \quad (8)$$

for the time evolution of the radius and the temperature of the shock, respectively. The integration constant is chosen in such a fashion that the passage of the nuclearite takes place at the time $t_0 = R_0 / v\sqrt{8}$. The thermodynamic description of the event is valid when R exceeds the mean free path l in the medium, corresponding to the time $t_1 = (l/R_0)^2 t_0$.

The expanding hot cylinder emits black-body radiation with a spectrum

$$-\frac{dp}{d\omega da} = \frac{\omega^3}{(2\pi)^2} \frac{1}{e^{\omega/T} - 1} \quad (9)$$

of power (p) radiated per unit frequency (ω) and per unit area (a). The total luminous energy radiated by the nuclearite per unit length of its trajectory is

$$\frac{dE_\gamma}{dx} = \int_{\omega_{\min}}^{\omega_{\max}} d\omega \int_{t_{\min}}^{\infty} dt 2\pi R(t) \frac{dp}{d\omega da} \quad (10)$$

The limits on frequency correspond to the window of transparency of the medium, and $t_{\min}$ is the greater of t_0 and t_1 . For a wide transparency window and under circumstances such that

$$T(l) \gg \omega_{\max} \quad (11)$$

—a condition that is satisfied for the cases we consider—equation (10) may be approximated by

$$\frac{dE_\gamma}{dx} \approx (6\pi^2\sqrt{2})^{-1} \omega_{\max}^{5/2} (m/n)^{3/2} \pi R_0^2 v^2 \quad (12)$$

The luminous efficiency is obtained by comparing equations (2) and (12),

$$\begin{aligned} \eta &\approx (6\pi^2\sqrt{2})^{-1} \omega_{\max}^{5/2} (m/n)^{3/2} \rho^{-1} \\ &\approx 2 \times 10^{-5} (\hbar\omega_{\max}/(\pi \text{ eV}))^{5/2} (\text{MW}/18)^{3/2} (3/n)^{3/2} (\rho_W/\rho) \end{aligned} \quad (13)$$

where $\pi \text{ eV}$ is a typical visible photon energy, MW is the mean molecular weight of the medium, $n=3$ is an estimate of the relevant number of excited subnuclear degrees of freedom and ρ_W is the density of water. In highly purified water ($\hbar\omega_{\max} = 3.75 \text{ eV}$ (see, for example, ref. 6 and refs therein)) we obtain $\eta \approx 3 \times 10^{-5}$, whereas in air, at atmospheric pressure, $\eta \approx 4\%$.

Note that equation (13) depends neither on the size R_0 of the nuclearite nor on its velocity provided that equation (11) which may be written

$$\frac{mv^2}{n} \gg (l/R_0)^2 \omega_{\max} \quad (14)$$

is valid. Recalling that the effective radius of a nuclearite is never less than $1 \text{ \AA}$, we find that equation (14) is satisfied in water for any nuclearite whose velocity has not been severely degraded. In air at sea level, equation (14) is satisfied by cosmic nuclearites exceeding a radius of $2 \times 10^{-7} \text{ cm}$ corresponding to a minimum mass of 10^{-5} g . Incidentally, equation (13) does not apply to ordinary meteors that ablate and are consumed before equation (12) is satisfied.

Some recent searches for proton decay can as well be adapted to searching for cosmic nuclearites. For example, the Irvine-Michigan-Brookhaven (IMB) proton decay detector⁵, consisting of 80 kt of purified water surrounded by phototubes at a depth of $1.7 \times 10^5 \text{ g cm}^{-2}$, detects the Cerenkov light produced

by relativistic charged particles as they traverse the detector. The water is highly transparent to visible photons with energies of 2.25–3.75 eV, and the device can detect events involving the production of at least 3×10^4 photons. From Fig. 2, it follows that only nuclearites heavier than 10^{-11} g reach the IMB detector. However, the nuclearite must not only reach the detector, but do so with a velocity greater than 30 km s^{-1} corresponding to a temperature of several thousand K, which means they must be heavier than $\sim 2 \times 10^{-11} \text{ g}$ for detection at IMB. On the other hand, to produce an event rate of $> 1 \text{ yr}^{-1}$ within the detector, the nuclearite flux must satisfy $F \geq 3 \times 10^{-5}$, with units (hereafter suppressed) $\text{km}^{-2} \text{ yr}^{-1} (2\pi \text{ sr})^{-1}$. If the IMB detector were programmed to detect slower moving nuclearites ($30\text{--}300 \text{ km s}^{-1}$), the region displayed in Fig. 1 could be explored and perhaps excluded within one year's operation.

Some experiments designed to search for cosmic magnetic monopoles consist of large surfaces of scintillator placed near the Earth's surface. Scintillating material is even more sensitive than water to the passage of nuclearites and can detect nuclearites with masses as small as 10^{-13} g , in the case of an unshielded detector at sea level. The region explorable with a planned detector^{7,8} of area $1,500 \text{ m}^2$ is shown in Fig. 1.

Nuclearites, like meteors, produce visible light as they traverse the atmosphere. Their rate of power dissipation, from equation (2), is $A\rho v^3$. Using the luminous efficiency given by equation (13), we can deduce an altitude-independent expression for their luminosity as a function of their mass

$$L = 1.5 \times 10^{-3} (M/1 \mu\text{g}) \text{ watt} \quad (15)$$

which is valid provided that equation (13) is satisfied. Because of the latter constraint, our analysis does not apply to nuclearites lighter than 10^{-5} g . The visual magnitude of an atmospheric nuclearite at a distance h from an observer is computed from equation (15) to be

$$\mathcal{M} = 10.8 - 1.67 \log_{10} (M/1 \mu\text{g}) + 5 \log_{10} (h/10 \text{ km}). \quad (16)$$

For example, the apparent magnitude of a 20-g nuclearite at a distance of 10 km is $\mathcal{M} = -1.4$, equal to that of the brightest star, Sirius.

Atmospheric nuclearites may easily be distinguished from ordinary meteors. Travelling at galactic velocities ($v \sim 250 \text{ km s}^{-1}$), they are much faster than meteors, which, being bound to the Solar System, move no faster than 72 km s^{-1} relative to the Earth. Moreover, meteors generally emit light only in the upper atmosphere ($\geq 100 \text{ km}$) where they ablate and disintegrate. We show below that the production of light by nuclearites is essentially a low-altitude phenomenon. Thus, the apparent angular velocity of a nuclearite is usually two orders of magnitude greater than that of a meteor.

The constraint imposed by equation (14) can be expressed as an upper limit to the altitude at which nuclearites effectively generate light. Using the fact that the scale height of the atmosphere is $\sim 8 \text{ km}$, we obtain

$$h_{\max} = 2.7 \text{ km} \ln (M/1.2 \times 10^{-5} \text{ g}). \quad (17)$$

Thus, nuclearites with a mass of 10^{-4} g produce their light at altitudes of less than 6 km whereas those with a mass of 10^4 g shine below 60 km. Evidently, the meteoric behaviour of nuclearites is essentially a phenomenon of the lower atmosphere.

From a point on the Earth, the flux corresponding to one annual atmospheric event is given by $F = 2/\pi h_{\max}^2$ at zenith angle smaller than $\pi/4$. (The factor of 2 reflects the difficulty in making daytime observations.) For a large domain of nuclearite masses, this expression which defines the excludable region shown in Fig. 1 is less than the maximum cosmic flux of nuclearites of equation (1). To what extent searches already performed have actually eliminated the excludable region is obscure to us. The largest nuclearite, compatible with the cosmic limit that is likely to be detected from one location during a year of observation, has a mass of $4 \times 10^4 \text{ g}$. The apparent magnitudes of hypothetical cosmic nuclearites range from $\mathcal{M} = +6$ for

nuclearites near 10^{-4} g to $M = -3$ for those with masses near the upper-mass cutoff.

Price *et al.*⁹ recently reported negative results of a search for magnetic monopoles having examined ancient mica for etchable trails of lattice defects produced by cosmic monopoles. Their data provide our only example of an established limit on the cosmic nuclearite flux, arguing that an energy loss, $dE/\rho dx \geq 2.5 \text{ GeV cm}^2 \text{ g}^{-1}$, in the form of nuclear recoil suffices to produce an etchable track. In the case of nuclearites, virtually all of the energy loss is of this kind. With their assumed mean mica burial depth of 5 km, we calculate from equation (9) that an etchable track is produced by any cosmic nuclearite with mass greater than 2.4×10^{-10} g. The mica sample has an area of 13.5 cm^2 and an estimated age of 0.5 Gyr. Thus, the minimal measurable nuclearite flux to which this experiment is sensitive is $F = 1.5$ and its excluded region is shown in Fig. 1.

Etchable tracks in mica are but one example of fossil traces that may be produced by nuclearites in rock. Any nuclearite more massive than 2.4×10^{-10} g can leave an observable track at great depth—an otherwise inexplicable linear astroblem. Neither meteorites, micrometeorites, natural radioactivity nor conventional cosmic radiation can produce such an effect. The tracks produced by nuclearites lighter than ~ 1 g are microscopic and require for their detection sophisticated searches on well-chosen materials in the spirit of the Price *et al.*'s mica experiment⁹. Larger nuclearites can produce visible astroblems. The energy dissipated by a cosmic nuclearite, given by equation (2), is sufficient to melt a cylinder of rock with a radius $R_M = v\beta^{-1/2}$, where $\beta \sim 800 \text{ J g}^{-1}$ is the energy required to heat and fuse 1 g of rock. Thus, $R_M \sim 400 R$. A 1-g nuclearite produces a fossil track with a diameter of $\sim 10^{-2}$ cm. The incidence of such fossils in 10^9 -yr-old rock is $\sim 1 \text{ cm}^{-2}$, if the upper limit on the cosmic nuclearite flux of equation (1) is attained. Still larger nuclearites may be associated with fossils consisting of crushed rock. From the fact that a 1-kt (TNT equivalent) nuclear test in granite produces a spherical crush zone of radius 30 m (ref. 10), we deduce that the radius of the crush zone associated with the passage of a large nuclearite is $\sim 2,000 R$. Thus, a 1-kt nuclearite produces a linear astroblem of diameter 4 m. The maximum possible frequency of such events is 10^{-2} km^{-2} per 10^9 years. We show in Fig. 1 the result of a hypothetical negative hunt for nuclearite fossils, terminating at nuclearite mass of 1.5×10^{18} g or radius 10 cm beyond which the maximum cosmic flux corresponds to less than one collision with the Earth in its lifetime.

The last effect of nuclearite passage that we shall consider is the possibility of nuclearite-induced epiliner earthquakes. Note that the diametric Earth passage of a 1-t nuclearite releases a total energy equivalent to a 50-kt (TNT equivalent) nuclear weapon. (At 30° zenith angle, this is reduced by a factor of 2; at 60° by a factor of 5.) Such nuclearites may collide with the Earth at a rate of no more than 1 yr^{-1} . At teleseismic distances, the seismic signal produced by a point source (weapons test or earthquake) is comparable in magnitude to that produced by a line source of the same total energy release. Indeed, the nuclearite may produce a larger signal, as its seismic efficiency when passing through the dense mantle may be larger than the $\sim 10^{-2}$ efficiency of a well-tamped nuclear shot in granite¹¹. Nuclearite seismic signals are readily distinguishable from other sources: their arrival times, characteristic of a linear sonic antenna, are vastly different from those of a point source. (Remember that the nuclearite traverses the Earth in < 1 min.) Surface waves should be absent, as most of the energy loss takes place in the mantle. Similarly, the production of shear waves is suppressed by a power of c/v , where c is the local sound velocity. Large epiliner earthquakes (body magnitude, $m_b > 5$) can perhaps be 'discovered' in existing seismological data. Thus, the excluded domain shown in Fig. 1 is bounded by $F \geq 10^{-9}$ corresponding to one event in the Earth per decade. The limit on signal size corresponds to an energy deposition equivalent to 1-kg TNT km^{-1} , or a nuclearite mass of 1 kg.

The experiments discussed here, as well as others (such as acoustic detection at sea and the effect of small nuclearites on

airglow) can severely constrain the hypothesis of a cosmic nuclearite flux. In particular, the explanation of the galactic missing-mass problem in terms of nuclearites smaller in size than several centimetres is a hypothesis that is subject to feasible (though highly interdisciplinary) experimental testing.

We thank G. Charkap, E. Farhi, G. Hansen, R. Jaffe, C. Jorgensen, R. E. McCrosky, P. McIntyre, L. B. Okun and L. Sulak for discussions and E. Witten for communicating his work to us prior to publication. This research is supported in part by the NSF under grant PHY82-15249.

Received 30 July; accepted 24 October 1984.

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A 164-day period in γ -ray bursts from GBS0526-66?

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The γ -ray burst history of GBS0526-66 is unique among observed burst sources¹ being that of the brightest burst on record (5 March 1979) and one of only two from which recurrent bursts (at least 15 of similar temporal and spectral pattern) have been observed (though much less intense² than the first), and the only one whose source direction coincides with that of a previously known object—its 0.1 arc min^2 error box lies within the supernova remnant N49 in the Large Magellanic Cloud³. We have found evidence for a pattern in the recurrence times of these bursts, showing an apparent period of 164 day, which we suggest reflects periodic accretion to a neutron star from an eccentric ($e \sim 0.9$) binary companion. We propose that this system drives two types of bursts: (1) the relatively frequent but weak recurrent bursts produced by either magnetospheric gating, or thermonuclear runaway, of periodically accreting matter around the neutron star, and (2) the much rarer intense bursts, like that of 5 March 1979, produced by tidally-triggered star quakes.

Continued monitoring by the Konus instruments aboard Venera 11 and 12 (from about 14 September 1978 to 27 January 1980) and Venera 13 and 14 (from about 16 November 1981 to 5 April 1983) has revealed² 15 weaker γ -ray bursts, following that of 5 March 1979, with the same celestial position, very similar spectra, and durations ranging from 0.1 to a few seconds. The fluences, S , of these weaker bursts, which varied from $\sim 1.5 \times 10^{-7}$ to $6.5 \times 10^{-6} \text{ erg cm}^{-2}$, were roughly proportional to their duration, and were orders of magnitude less than the $1.3 \times 10^{-3} \text{ erg cm}^{-2}$ observed from the 5 March 1979 burst.

The most striking feature of these recurrent bursts, however, is that their recurrence times, Δ —the time elapsed since the previous burst—show a very high degree of ordering. This is clearly seen in both Table 1 and Fig. 1 (bottom) for the observations of bursts by Venera 13/14 which were of higher sensitivity than those of Venera 11/12, and provided a much more complete sample. Starting from the first burst on 1 December 1981, the recurrence times increased steadily over the next three bursts. Then, on 23 April 1982, the recurrence time abruptly shortened to 0.5 day, and then once again increased over the next six successive bursts. The random probability of nine successive values occurring in one or two sets of any combination of lengths,

ordered with either rising or falling values, is twice the sum of the binomial coefficients, $\sum_{s=1}^8 9!/(9-s)!s!$ divided by the total number of combinations, $9!$, or 0.0028. We must multiply this probability by three, as there are three sets of nine contiguous values in the complete group of 11 values. Thus, we suggest that the chance of this ordering being non-random is 99.2%, or significant at the 2.6σ level.

Moreover, we see from Fig. 1 (bottom) that the values of the recurrence times for the six bursts, beginning with the second burst on 23 April 1982, are roughly proportional to the time of occurrence measured from 23 April 1982. The three previous recurrence times also show an almost linear increase with time. We propose that this ordered pattern of recurrence times may repeat with a regular period. If so, then the observations before 23 April 1982 would suggest that the period is ≥ 140 day, and the later observations would suggest that it is ≤ 350 day. As the two half-day recurrences on 5–6 March 1979 and 23 April 1982 should occur at about the same phase, the period should also be roughly an integral divisor of the 1,145-day interval between them. These constraints limit the possible periods to 143, 164, 191, 229, and 268 day. Folding the data on these periods, we find that the best qualitative fit for all the data is obtained with the period of 164 ± 0.75 day; the best linear relationship between recurrence time and phase is shown with the folded data in Figs 1 and 2 (bottom).

This simple linear relationship agrees qualitatively with all but the event of 31 December 1982, which has a recurrence time about twice that expected. This discrepancy, however, may be owing to a missing burst in October or November 1982, resulting from the fluence being below the detector threshold, or the detector system being temporarily inoperative. The latter possibility would seem likely, if the operating schedule of Venera 13/14 was similar to that of Venera 11/12 on which the operating time of the Konus experiment dropped from almost 90% of the time, during the first half of the mission, to roughly 50%, during the final few months⁴. As the Venera 13/14 threshold appears to be at least a factor of three lower^{2,5} than that of Venera 11/12, the smaller number of recurrent bursts detected in 1978–80 compared with 1981–83—that is, three as opposed to twelve—may result simply from detector threshold differences. For bursts greater than the Venera 11/12 threshold ($> 5 \times 10^{-7}$ erg cm⁻²), there is no evidence for a change in the rate of recurrence, as the same number, were observed during both missions. Thus, both sets of Konus observations could be consistent with regular bursting.

If we also consider the equivalent mean flux (S/Δ) versus time, we see in Figs 1 (top) and 2 (top) that most of the observed emission comes at intervals of roughly 164 day or a multiple thereof. The highest fluxes are strongly peaked close to phase 0.0, and all fluxes greater than twice the minimum of 2.3×10^{-14} erg cm⁻² s⁻¹ occur within a phase interval of 0.33. Moreover, this latter phase interval contains 96% of the time-integrated flux, or fluence, from all bursts observed following that of 5 March 1979.

Assigning a numerical probability to the existence of a 164-day periodicity is very difficult for the following reasons. First, a simple power-spectral analysis of occurrence times would not be expected to yield significant results, because the 16 events were spread over an interval of 1,500 day that included a 1,000-day gap and, thus, only a limited number of long periods could be sampled. If we use the technique of Ferraz-Mello⁶, however, which is designed to accommodate such data gaps, a simple sinusoidal 164-day period is only significant at the 15% level, because the distribution of occurrence times is far from sinusoidal, requiring many higher harmonics, which the limited data cannot resolve. A power-spectral analysis of the equivalent mean flux will also be insensitive to any specific period, as the almost 'delta-function' emission of 0.5-day duration dominates the power spectrum and causes almost equal power to appear at all frequencies.

Our analyses, however, clearly suggest that the highly ordered pattern of recurrence times is non-random at the 2.6σ level and

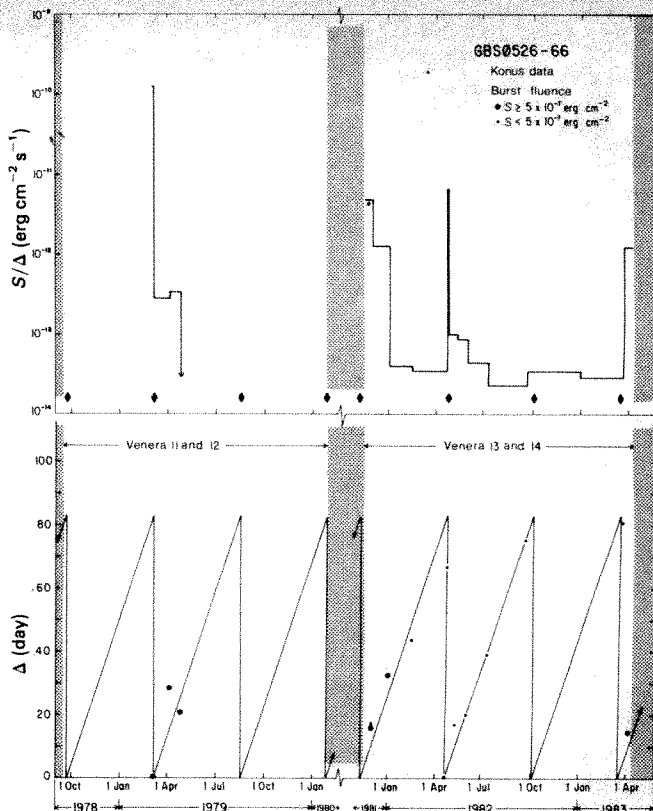


Fig. 1 The distribution of the burst recurrence times, or time between bursts, Δ , shown at the occurrence time of each burst (bottom), and the equivalent mean flux, or the burst fluence divided by the recurrence time, S/Δ , shown for the interval preceding each burst (top). Also shown are diamonds marking 164-day periods and a least-squares linear fit to the recurrence times within that period. All burst fluences greater than the Venera 11/12 threshold of $\sim 5 \times 10^{-7}$ erg cm⁻² are designated by large dots, and those fluences below are shown as small dots.

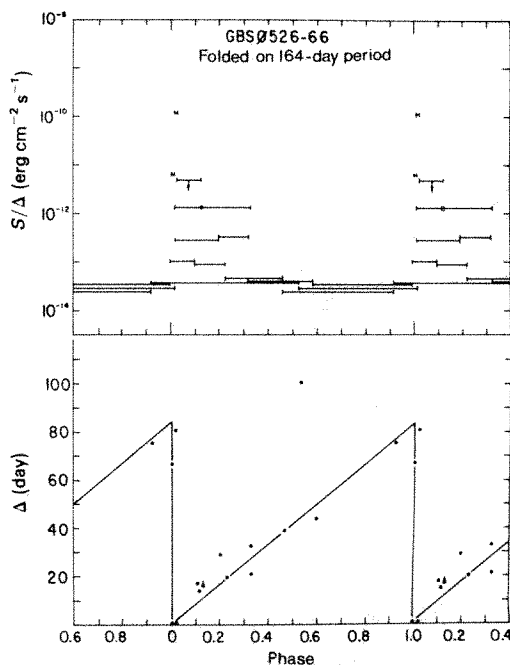


Fig. 2 The phase dependence of the burst recurrence time (bottom), and the equivalent mean flux (top), for a 164-day period. The solid line in the bottom panel represents the best linear fit to the recurrence-time distribution.

Table 1 Bursts from GBS0526-66 observed by Konus²

Burst date (year, month, day)	Julian day (-2443900)	Δ (day)	S (10^{-6} erg cm $^{-2}$)	Phase
79 03 05	38.16	—	1300	0.02
79 03 06	38.76	0.60	6.5	0.02
79 04 04	67.53	28.77	0.7	0.20
79 04 24	88.27	20.74	0.6	0.33
81 12 01	1039.89	>16	6.6	0.13
82 01 02	1072.22	32.33	3.6	0.33
82 02 15	1115.94	43.72	0.15	0.59
82 04 23	1182.66	66.72	0.2	0.00
82 04 23	1183.19	0.53	0.3	0.00
82 05 10	1200.06	16.87	0.15	0.11
82 05 30	1220.03	19.97	0.15	0.23
82 07 08	1258.93	38.90	0.15	0.46
82 09 21	1334.28	75.35	0.15	0.92
82 12 31	1434.68	100.40	0.3	0.54
83 03 21	1515.44	80.76	0.2	0.03
83 04 05	1529.83	14.39	1.6	0.12

Phase based on 164-day period and phase-zero at first burst on 82 04 23.

that this pattern exhibits a 164-day periodicity: burst occurrences are highly asymmetrical—half of them within a very small portion (~ 0.13) of the cycle; $\approx 96\%$ of the recurrent burst energy is emitted within a third of the cycle, and the time between bursts increases almost linearly with phase.

We now consider the possible causes of this pattern of recurrent bursts. GBS0526-66 is generally thought to be a magnetic neutron star^{7,8}. Consistent with this, the cause of the intense burst of 5 March 1979 has been variously attributed to a neutron star quake⁹, thermonuclear runaway of accreted matter on the neutron-star surface¹⁰, or cometary impact¹¹. The three much weaker bursts observed within two months of the 5 March 1979 burst were thought to have been aftershocks or other events resulting from it.

We suggest, however, that the recurrent bursts are separate, although not entirely unrelated, continuing phenomena, occurring both before and after the 5 March 1979 burst. In particular, we propose that this periodic, but asymmetric, pattern of recurrent bursts may result from either thermonuclear runaway or magnetospheric gating of matter periodically accreting around the magnetic neutron star from a binary companion with a 164-day period and a highly eccentric ($e \sim 0.9$) orbit. Both the asymmetric rate of burst occurrence and the increasing recurrence time as a function of phase can be understood if the mass transfer from the companion is essentially limited within a time of the order of a day around periastron. Then the rate of gas accretion to the magnetosphere of the neutron star would be greatest at the time of periastron passage and decrease with time as gas with greater initial angular momentum eventually falls in. With such a decreasing rate of accretion, the individual bursts themselves could result from the filling of some reservoir to a critical mass, which then becomes unstable, producing a burst. The recurrence times between successive bursts will then increase until a new accretion episode is initiated at the next periastron passage 164 day later. The reservoir could be either the accumulation of matter on the surface of the neutron star until it undergoes thermonuclear runaway to produce the γ -ray burst¹⁰, or the accumulation of matter in an accretion disk above the magnetopause, where it remains until an instability develops, dumping it on the surface of the star to produce the burst¹².

The eccentricity of the binary orbit can be estimated from the ratio of the time, p , required for passage through a radian at periastron and the 164-day orbital period, P , by $e \approx 1 - (4\pi p/P)^{2/3}$. If the periastron passage is about 0.5 day—the minimum observed burst separation—then the eccentricity, $e \sim 0.89$, and the size of the Roche lobe would vary by a factor of 20 over the orbital period. Optical searches (F. Cordova, personal communication) show that the GBS0526-66 error box is

empty down to the background brightness of the nebula, giving a limiting apparent magnitude for a companion of ~ 18 m (visual). At the distance (55 kpc) of the coincident supernova remnant in the Large Magellanic Cloud, this limits the mass of a main-sequence companion, for instance, to $\leq 5 M_{\odot}$. We suggest that it is during this brief, but close, approach that the bulk of the matter, accreted over an entire orbital period, flows into the potential well of the neutron star.

We might also expect that if a burst happened to occur on the neutron star close to periastron passage, then the flash heating of the atmosphere of the companion might greatly boost the amount of matter transferred at that time. The efficiency of this process will depend strongly on the orbital phase, as the heating would be proportional to the solid angle subtended by the companion and would vary by roughly three orders of magnitude over the orbital period. The occurrence of bursts as close together as 0.5 day and the tendency for brighter bursts to occur early in the phase may in fact require just such a flash-boosted accretion to provide the very brief but enormous accretion rates needed on those occasions.

The soft X-ray observations place only weak constraints on the accretion rates. Einstein observations of the GBS0526-66 region, both before and after the 5 March 1979 burst, set a 3σ upper limit of $\sim 2 \times 10^{-12}$ erg cm $^{-2}$ s $^{-1}$ on the excess flux of soft (0.5–4.5 keV) X rays observed on 12 April 1979 between the recurrent bursts of 4 and 24 April 1979¹³. This can be compared with the steady flux expected from the accreting matter in magnetospheric gating and thermonuclear runaway models. For a magnetospheric gating process, the expected average radiation flux from matter accreting around the magnetopause at the time of the Einstein observation should be about three orders of magnitude below the soft X-ray limit. But in the thermonuclear model a simple estimate of the bolometric flux at that time suggests that it should be at least $\sim 1 \times 10^{-11}$ erg cm $^{-2}$ s $^{-1}$, which is several times higher than the 3σ upper limit in the 0.5–4.5 keV band. With more detailed calculations, however, the thermonuclear model may still be consistent with the observations.

Finally, we suggest that the accretion of matter from the binary companion may build up stresses within the neutron star that could eventually be released in very powerful quakes, providing energy for much larger but rarer bursts, such as that of 5 March 1979. We may also expect that these bursts would occur preferentially near periastron passage where they could be triggered by the much larger tidal stresses at that time.

In conclusion, we suggest that the pattern of recurrent bursts from GBS0526-66 displays a 164-day periodicity and, if confirmed, this represents the first periodicity seen in γ -ray bursts and the first evidence for a binary companion around a burst source. Further monitoring of this source and study of existing observations are needed to confirm the existence of this periodicity. The strongly asymmetric distribution of burst rates would suggest that there are preferred times for the occurrence of a burst.

We thank Geoff Hueter for helpful discussions. This work was supported by NASA contract NAS 8-27974 and NASA grant NSG 7541.

Note added in proof: Since submitting this paper, we have been informed (H. Pedersen and K. Hurley, personal communication) that an optical flash occurring at phase 0.001 by our ephemeris (Table 1) had been detected at the European Southern Observatory-La Silla by their 50-cm telescope (now published¹⁴), seemingly confirming the 164-day period. We predict that periastron passage will occur on 1 January 1985 (± 3 day).

Received 14 May; accepted 6 November 1984.

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Lightning-induced electron precipitation

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The broadband very low frequency (VLF, 0.3–30 kHz) radiation from lightning propagates in the Earth–ionosphere cavity as impulsive signals (spherics) and in the dispersive plasma regions of the ionosphere and magnetosphere it propagates as tones of descending or rising frequency (whistlers)¹. VLF radio waves propagating in the magnetospheric plasma scatter energetic electrons by whistler-mode wave–particle interactions (cyclotron resonance) into the atmosphere^{2–6}. These electrons, through collisions with the atmospheric constituents, cause localized ionization, conductivity enhancement, visual and ultraviolet light emissions, and bremsstrahlung X rays. We have reported previously on the precipitation of energetic electrons from the radiation belts by the controlled injection from the ground of VLF radio waves^{7,8}. Here we report the first satellite measurements of electron precipitation by lightning. The measured energy deposition of these conspicuous lightning-induced electron precipitation (LEP) bursts ($\sim 10^{-3}$ erg cm⁻²) is sufficient to deplete the Earth's radiation belts and to alter subionospheric radiowave propagation (≤ 1 MHz). A one-to-one correlation is found between ground-based measurements of VLF spherics and whistlers at Palmer, Antarctica, and low-altitude satellite (S81-1) measurements of precipitating energetic electrons.

Detailed measurements of the pulse shape, spectrum, and pitch angle distribution of LEP events have not previously been obtained nor have direct satellite or ground-based measurements of LEP events been obtained within the plasmasphere where most VLF whistler activity occurs⁹. The plasmasphere (average magnetic invariant latitude $\leq 60^\circ$) is characterized by cold plasma densities of 10^2 – 10^4 electrons cm⁻³ and is the region of the magnetosphere which contains the bulk of the radiation belts. The only *in situ* measurement of lightning-induced electron precipitation that we are aware of was reported by Rycroft¹⁰ using rocket data. He observed a single electron burst event having the proper time relationship to an associated whistler and explained the observation as a gyro resonant interaction between ~ 100 keV electrons and a $\frac{1}{2}$ -hop whistler taking place in the magnetospheric equatorial plane. Our *in situ* satellite observations confirm this initial rocket measurement by establishing a one-to-one correlation with a series of strong electron burst events and clarify the details of the precipitating electron temporal behaviour. Electron precipitation due to whistler-triggered emissions outside of the plasmasphere has been observed with balloon-borne X-ray detectors⁶ and ground-based photometers^{11,12}. Indirect evidence of LEP events within the plasmasphere has been derived from amplitude and phase perturbations in VLF signals propagating in the Earth–ionosphere waveguide^{13,14}.

High sensitivity measurements and fine-resolution energy spectra ($2 < E < 1,000$ keV) of the prominent LEP bursts were obtained with a cooled solid-state spectrometer array¹⁵ included as part of the stimulated emissions of energetic particles (SEEP)

experiment on the three-axis stabilized, low-altitude (~ 230 km) S81-1 satellite. The trapped energetic (TE) electron spectrometer ($\pm 20^\circ$ field of view) was aligned perpendicularly to the orbit plane and during these observations was at an angle of 89° to the local magnetic field line. The TE detector had a geometrical factor of 0.17 cm² sr and was cooled (-120°C) to achieve a system noise resolution of 1.2 keV FWHM. An identical detector was positioned to observe electrons with central pitch angles (α) of 52° . The medium energy (ME), precipitating electron spectrometer ($\pm 30^\circ$ field of view) was aligned to the zenith direction and during these observations was at an angle of 25° to the local magnetic field line. The ME geometrical factor was 2.47 cm² sr.

Seven LEP events recorded on 9 September 1982 with the SEEP experiment TE particle spectrometer are shown in Fig. 1 (A–G) with the simultaneous VLF spectra received at Palmer, Antarctica ($L \approx 2.3$). These LEP event signatures are interpreted as follows. The whistler wave in passing through the magnetosphere from north to south alters the pitch angles of energetic trapped electrons which are moving northward and can, therefore, resonate with the VLF wave. This interaction reduces the pitch angles of some of the electrons, lowers their mirror points below the satellite altitude, and produces the first electron pulse of the event. Some of these electrons are then magnetically reflected and some are scattered by the atmosphere, resulting in an electron bunch moving to the Southern Hemisphere where the lower mirroring altitude (due to the South Atlantic anomaly) causes the electrons to encounter the atmosphere. Some electrons are backscattered by the atmosphere and return to the Northern Hemisphere where they are observed as the second pulse in the event. Subsequent reflections and backscattering in the Northern and Southern Hemispheres produce the train of pulses of diminishing intensity which makes up the individual events shown in Figs 1 and 2. These measurements were made 3 days after the strong magnetic storm ($D_{st} = -297$) of 6 September 1982. Magnetic storms of this intensity are known to inject electrons which diffuse into the slot region ($2 < L < 3$) of the radiation belt several days after the storm onset^{16,17}.

In the strong LEP events A, D, and E, electron fluxes are observed to increase rapidly in strength, about 100 times background, in < 200 ms. The envelopes of the individual pulses then decay relatively slowly to background levels over several seconds. Event G is a factor of 10 above background and event F about three times background. Events B and C are relatively weak on the integral energy display of Fig. 1 but are more prominent in the differential energy spectrum ($120 < E < 140$ keV). The reason why the multiflash LEP event E does not show echo pulses may be due to the superposition of four closely spaced LEP bursts. The flux measured with the ME detector ($\alpha \approx 25^\circ$) is a factor of ~ 10 less than the flux measured with the TE detector ($\alpha \approx 89^\circ$) indicating an anisotropic pitch angle distribution peaked near 90° at the satellite altitude. VLF spectra of lightning generated spherics and whistlers were detected in the conjugate hemisphere at Palmer Station, Antarctica, (65° S, 64° W) and are shown in Fig. 1a. The uniform transition in VLF wave intensity at ~ 1.9 kHz indicates a well defined Earth–ionosphere waveguide cutoff frequency. The scaled spherics and whistlers are shown beneath the VLF spectrogram. The dashed portion of a scaled whistler curve is extrapolated based on the observed solid portion and the known properties of more completely defined events such as F. The conjugate of the SEEP satellite (60° S, 98° W) is 34° to the west of Palmer and thus the VLF spectrogram intensity at Palmer is not simply related to the magnetospheric whistler intensity nor to the flux of precipitating electrons.

Whistler-dispersion techniques¹ are used to identify the spherics that precede the delayed whistler signal on the VLF spectrogram. Event E has four similar whistler traces within a 1-s period and is consistent with multiflash lightning. Event C also has two lightning flashes associated with it. An important observation is that the spherics precede the peak of each LEP event by the expected time interval (≈ 0.4 s) for all seven cases.

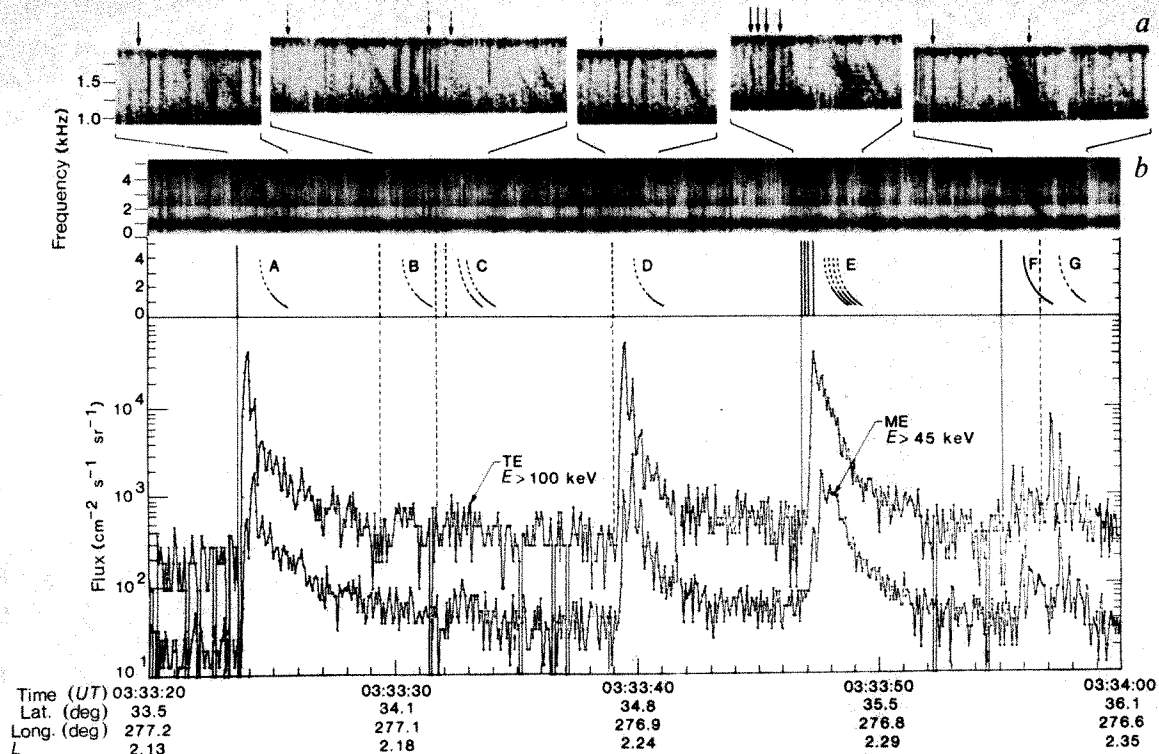


Fig. 1 Energetic electron measurements obtained with the S81-1 satellite are correlated one-to-one with concurrent ground-based VLF whistlers at Palmer Station, Antarctica (65°S , 64°W , $L \approx 2.3$). Panels *a* and *b* show the VLF spectrograms and the scaled whistlers and spherics. The uppermost insets show the Palmer VLF data with an expanded time scale for the seven LEP events. The strong energetic electron bursts labelled A, D and E have peak fluxes that are 100 times background levels. The sampling interval for the energetic particle measurements is 64 ms. The solid lines and arrows represent spherics that were directly identified on these (and other) records, while the dashed lines represent time estimates based on evidence of essentially identical dispersion properties in all events.

Further evidence for the triggering of precipitation by lightning is given by the LEP pulse shapes and spectra. In the pulse shapes of LEP events A, D, F and G, repetitive pulses of constant period and decaying amplitude that follow the LEP peak are conspicuous. Figure 2 shows LEP events F and G on an expanded scale with the associated whistler signals and the subionospherically propagating signals from the transmitter NAA as received at Palmer. The detailed characteristics of the LEP events shown in Fig. 2 include: (1) the rapid rise of electron flux; (2) the subsequent and relatively slow decay of the flux; (3) the in-phase and repetitive pulses on the TE and ME detector (labelled 1–3 for event F and 1–5 for event G); (4) the greater intensity of the near 90° pitch angle flux (TE) compared with the near 0° pitch angle flux (ME); and (5) the weak or completely absent first pulse on the ME spectrometer (indicating a high ratio of drift-loss-cone to direct bounce-loss-cone flux) compared with the subsequent pulses (labelled 2–5 for event G).

A pulse period of 0.32 s is associated with pulses 1–7 of event A. This period agrees with the bounce time of relativistic electrons (~ 150 keV) echoing between conjugate hemispheres at $L = 2.1$. For 175-keV electrons, the relativistic velocity is $0.67c$ and for 125 keV electrons $0.60c$, where c is the velocity of light. As the velocity approaches c the bounce period becomes less sensitive to electron energy variations. For the above energy difference, about five bounce periods (pulses) can occur before the 175-keV electrons are 180° out of phase with the 125-keV electrons. The pulse period of LEP event G at $L = 2.3$ is 0.38 s. This longer period relative to LEP event A agrees with the longer path length of relativistic electrons echoing between conjugate hemispheres at $L = 2.3$ instead of $L = 2.1$.

Examination of the electron energy spectra of LEP events A–G indicates a prominent but broad peak in electron energy between 80 and 200 keV that decreases in energy with increasing L . From comparison of the travel time characteristics of the 9 September 1982, whistlers with similar but more completely defined events recorded on another day at Palmer when whistler-associated precipitation was observed¹⁸, the equatorial electron

density at $L = 2.1$ is estimated to have been $3,200$ electrons cm^{-3} . Assuming ducted propagation the VLF wave frequency would be ~ 4 kHz based on an equatorial gyroresonant interaction with 150 keV electrons. This frequency is in the upper region of the more broadly defined whistlers, such as event F.

Figure 2a shows a small perturbation in the signal received at Palmer from NAA (propagating in the Earth-ionosphere waveguide) which is coincident with the strong whistler and weak LEP burst F. The raw data show the 17.8-kHz signal intensity using a 300-Hz bandwidth filter. Also shown are the smoothed curves of the raw data (dashed lines) outside of the rapid transition interval (± 0.5 s) that were obtained using a 2.4 s averaging filter. The amplitude of the NAA transmitter signal received at Palmer exhibits a fast decrease (< 1.5 s) followed by a slow recovery (~ 10 s). Such a signal perturbation or 'Trimpi' event is associated with lightning-induced electron precipitation which modifies the ionosphere at the ~ 80 km reflection altitude^{13,14}. Considered in isolation, this event is weak because of atmospheric noise at the NAA frequency. However, it is similar in form to a series of stronger events that occurred within several minutes preceding and following the period of satellite data. The strong whistler event F and associated Trimpi event recorded at Palmer are consistent with a relatively strong LEP burst occurring near Palmer. For the other six relatively weak whistler signals no Trimpi events were detected although strong electron precipitation is evident in the SEEP detectors for events A, D, E and G. The simultaneous electron precipitation at the SEEP satellite and near Palmer (Trimpi) which are separated in longitude by 2,000 km, for event F, suggest that a single lightning flash can precipitate electrons at two widely separated locations.

The electron precipitation energy fluxes are estimated to be about 10^2 – 10^5 times the wave energy flux at the equatorial plane, indicating that only a little VLF signal energy is required to perturb the relativistic electrons near the edge of the loss cone enough to cause precipitation³. The energy fluxes reported here are consistent with suggestions that whistlers may cause a

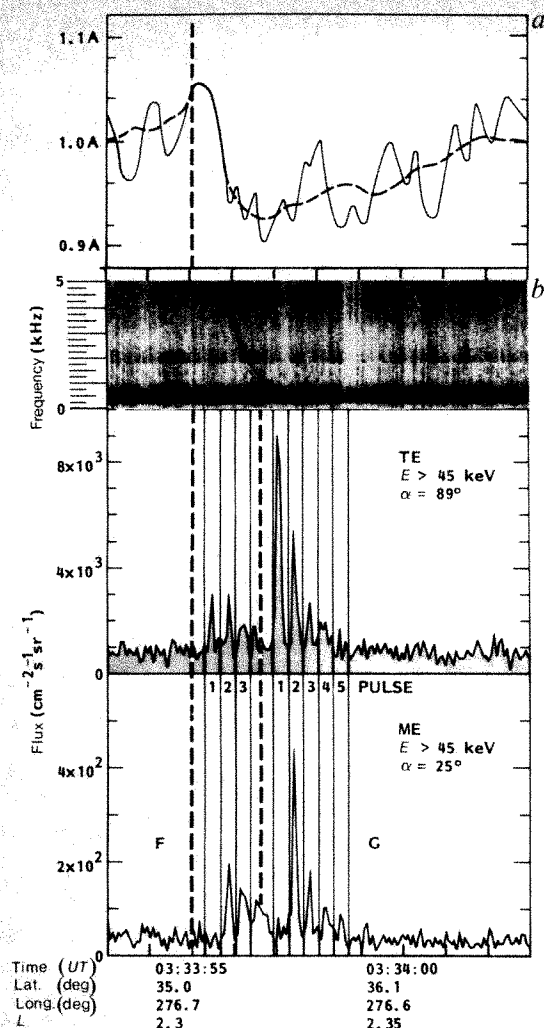


Fig. 2 Expanded view of lightning-induced electron precipitation (LEP) events F and G. The repetitive pulses of constant period labelled 1 to 5 are consistent with the echo period for electrons mirroring between conjugate hemispheres. The vertical dashed line indicates the calculated time of the lightning flash. Panels a and b show the simultaneous whistler spectrogram and the amplitude of signals received at Palmer from NAA.

significant loss of radiation belt electrons^{3-5,19,20,22}. Assuming that the slot regions of the radiation belts are initially filled after a magnetic storm with an omnidirectional flux of 10^8 electrons $\text{cm}^{-2} \text{s}^{-1}$ for $E > 100$ keV at $L = 2.3$, a single LEP burst (such as event D) can empty $\sim 0.001\%$ of the belt in the region covered by the burst magnetic field lines.

The observations of LEP events provide direct evidence of an important coupling mechanism between terrestrial lightning and relativistic radiation belt electron precipitation. Further study should clarify details of the wave-particle interaction since wide ranges of VLF signal strength and frequencies are present. The global mapping of LEP events can also provide an insight into the penetration of wave energy through the ionosphere and its propagation in the magnetosphere. The role of LEP events in the overall loss rates of trapped electrons is uncertain, because the frequency of occurrence of these events and their relative importance in comparison, for example, with plasmaspheric hiss²¹ is not well known. However, a substantial amount of electrons can be removed in a single event, and additional electron losses can be expected to result from an enhancement of the effective pitch angle diffusion coefficient by whistler waves.

The Lockheed Palo Alto Research Laboratory portion of the SEEP experiment was sponsored by the Office of Naval Research (contract N00014-79-C-0824). Launch and orbital support were

provided by the Air Force Space Test Program Office. Much of the data analysis was performed under the Lockheed Independent Research Program. We thank Dr D. P. Cauffman for program management of the satellite payload; the payload system engineer, Mr S. J. Battel; and Dr D. W. Datlowe for help in processing the data. The Stanford University effort in SEEP was supported by ONR grant N00014-82-K-0489 and by the Division of Polar Program of the NSF under grant DPP82-17820 for the Palmer Station VLF program.

Received 21 June; accepted 3 October 1984.

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A new radiation source for the infrared region

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Spectroscopy in the far-infrared and millimetre-microwave regions has always been hampered by the lack of intense broad-band sources. After several predictions¹⁻⁴ suggesting the possibility of obtaining improved infrared fluxes from an electron source, the Daresbury (SERC) synchrotron storage ring (SRS) has now been shown to produce an intense and very bright source of infrared photons. The infrared port (IR-13(1)) at Daresbury will provide scientists interested in the microscopic structure and dynamics of materials with a radiation source having several unique properties. As well as these properties, we describe here the work that has been done, since January 1984, in characterizing the infrared beam and suggest some wide-ranging potential experimental applications.

Because the photon beam is composed of electromagnetic radiation emitted by a highly relativistic electron beam⁵, the radiation is highly collimated in the forward direction and is strongly linearly-polarized in the orbit plane. Furthermore, it provides a well-defined train of pulses of radiation⁶ of ~ 200 ps in width with a period of either 320 ns (for a single electron bunch) or 2 ns (for 160 electron bunches). The pulse width and shape are entirely independent of frequency, and the inherent photon noise level is expected to be very low compared with that of black-body sources. The beam lifetime is 8-10 h at 2 GeV electron energy. Although the SRS is more intense (in terms of photons emitted per second) than a black-body emitter only in the far-infrared region (below $\sim 50 \text{ cm}^{-1}$), its other advantages—especially its brightness (watts per unit area-solid angle) and its precise time structure—are manifest throughout the infrared.

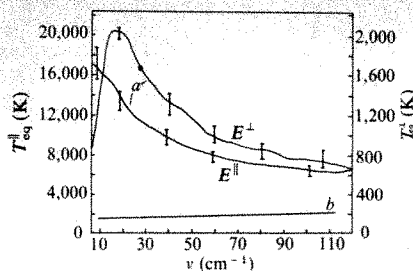


Fig. 1 Equivalent black-body temperatures ($E^{\parallel}$ and $E^{\perp}$) measured for the SRS source (a) (left-hand side, scaled to a 350-mA current) and the mercury lamp (b) (right-hand side) in the region 5–120 cm^{-1} . The effective aperture for both sources was 9 mm.

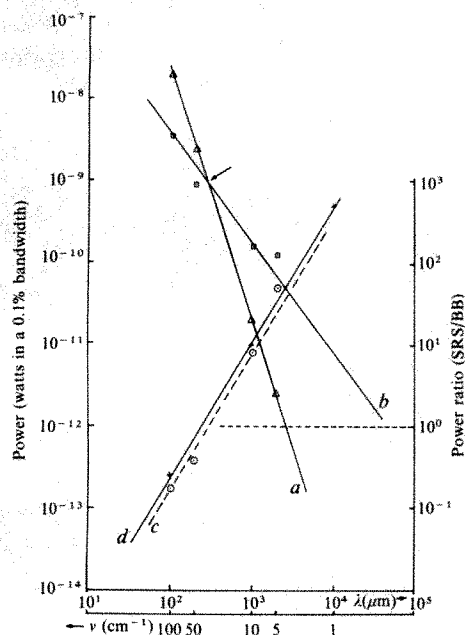


Fig. 2 Comparison of SRS and black-body output powers and power ratios: a, black-body curve at 1,500 K with a 3-mm stop ($A\Omega = 0.045 \text{ cm}^2 \text{ sr}$) (Δ); b, measured SRS power output at 350-mA current and $\pm 20 \text{ mrad}$ vertical collection ($\square$); c, power ratio measured at Daresbury in May 1984 ($\circ$); d, theoretical power ratio⁹ for a total vertical-angle collection and 350 mA (+).

Use of a Martin-Puplett interferometer⁷ operating in the 1–140- cm^{-1} (30–4,200 GHz) region in the radiometric mode⁸ has enabled us to measure directly the power and brightness ratios relative to those of a conventional far-infrared source (in this case, a high-pressure mercury arc lamp). Figure 1 shows the brightness measurements ($B(\bar{\nu}) = 2k\bar{\nu}^2 c T_{\text{eq}}$ watts per area-solid angle per bandwidth) made with a source aperture of $\sim 9 \text{ mm}$ —ensuring that the same étendue ($A\Omega$, area-solid angle product) is used in both cases. (The $B(\bar{\nu})$ data have been scaled to the 350-mA current for which our original computed SRS emission^{8,9} was calculated; such currents are easily achieved, although at rather lower electron energies than the standard 1.8 or 2.0 GeV.) The brightness advantage is more than 20:1 at 10 cm^{-1} for a 9-mm aperture. Figure 2 compares the powers ($P(\bar{\nu}) = B(\bar{\nu})A\Omega\Delta\bar{\nu}$) and power ratios with those predicted by Duncan and Williams⁹. For these measurements a mercury lamp aperture of 3 mm was used and $(A\Omega)_{\text{SRS}} \neq (A\Omega)_{\text{Hg}}$. The power ratios are within a factor of five of the theoretical values⁹. The source provides at least an order of magnitude more power at 10 cm^{-1} than a conventional mercury arc lamp, further increasing below 10 cm^{-1} , probably because of coherence effects¹⁰ found when the photon wavelength and the electron bunch length become comparable. Far-infrared lasers¹¹ do, of course, provide output powers in excess of these. The great advantage

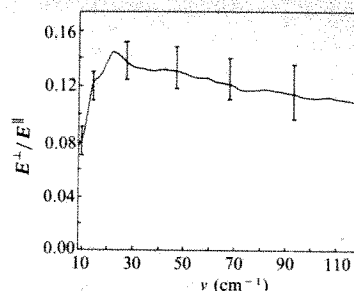


Fig. 3 The integrated polarization ratio $E^{\parallel}/E^{\perp}$ measured for the SRS source in the 8–120- cm^{-1} region (both normalized to a 350-mA current).

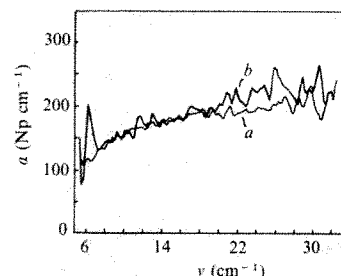


Fig. 4 Comparison of signal-to-noise ratios for a 100- μm sample of liquid water (298 K) in the 4–35- cm^{-1} region, using the SRS source (a), current $\sim 120 \text{ mA}$, and a mercury lamp (b).

of the SRS source is its tunability, in principle from 500 MHz upwards^{1–4}; in practice, we have detected radiation with frequencies down to 30 GHz (1 cm^{-1}) with our present interferometer. No other source provides such continuous radiation throughout the infrared and microwave regions. Also the SRS source has a much smaller pulse width than that of submillimetre lasers, which should give access to faster time-dependent processes^{5,6}, and it is $\sim 86\%$ polarized in the parallel (orbit plane) direction in the 10–100- cm^{-1} region (Fig. 3).

The SRS should provide an ideal infrared source for the following types of experiment:

(1) Experiments requiring enhanced signal-to-noise ratio in the far-infrared/microwave region. For example, we have used the combination of extra photon flux and superior noise characteristics to measure the power absorption coefficient of liquid water (through a pathlength of 100 μm) in the range 4–55 cm^{-1} with signal-to-noise ratio enhancement of ~ 3 (Fig. 4). Such measurements are urgently needed for accurate characterization of water^{12,13} in this important communications region.

(2) Experiments attempting to study the wide range of phenomena occurring in the important spectral gap between the infrared and microwave regions, including the modelling of molecular orientational relaxation processes in polar liquids¹⁴, the study of plasma frequencies in conducting polymers¹⁵, and the study of coherent phenomena^{16,17} and microwave radiation damage¹⁸ in biological systems.

(3) Experiments requiring sub-nanosecond time resolution in the middle and far-infrared regions include those for many important electronic^{19,20}, chemical²¹ and biochemical^{5,6} processes to which the SRS could be applied. One application already proposed by M. Chamberlain (personal communication) is to look directly at the electron kinetics of photothermal ionization in III–V semiconductors using submillimetre (45 cm^{-1}) radiation.

(4) Experiments requiring a photon beam with low étendue ($A\Omega$), either where the sample is small (for example, a single crystal or the small aperture in a high-pressure diamond anvil cell²²) or because the beam must be coupled to a surface whose étendue is also small (as in reflection-absorption infrared spec-

troscopy²³⁻²⁵). Étendues of the order of 10^{-3} cm² sr are obtained for the SRS⁹ at 50 cm⁻¹ and this matches the typical surface experiment²³ quite well. This characteristic of the SRS source should be important, for example, in studies of molecules adsorbed on catalytic (metal) surfaces or in studies of lubricating fluids at high pressures²⁶.

(5) Experiments making use of the beam polarization, which is important, for example, in the study of molecules on surfaces²³⁻²⁵, in the elucidation of optical properties of single crystals²⁷ and in the measurement of birefringence in liquid crystalline materials²⁸.

Besides the intrinsic interest in the study of photon emission from stored electron 'bunches', we anticipate that such novel

experimental features will be relevant in many areas of scientific investigation, having potential applications in the chemistry, physics and biology of materials.

We thank the SRFC of the SERC for continuous funding of this project; Professor D. H. Martin for providing much equipment; the scientists, engineers and support staff of the Daresbury Laboratory involved in the project, especially Drs Ian Munro, John West and Margaret Kelly, Messrs Bill Smith and Phil Moore, and Dr P. Ridley and his team. We also thank Dr W. D. Duncan who ran the initial stages of the project. T.N. thanks Dr I. Munro for an invitation to the Daresbury Laboratory, and also Professors K. Kimura and H. Inokuchi of the IMS at Okazaki (Japan) for their encouragement.

Received 23 July; accepted 18 October 1984.

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Photocharging of thin films of silver iodide and its relevance to the Daguerre photographic process

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In the Daguerre photographic process¹⁻³, a polished silver-coated plate is exposed to iodine vapour, thereby forming a very thin film of silver iodide, usually about 30-nm thick⁴. After exposure, the image is developed by mercury vapour, which condenses preferentially where light has fallen, amalgamating with silver liberated from the silver iodide (and possibly with the substrate). The remaining silver iodide is dissolved with an aqueous photographic fixing solution (sodium thiosulphate). When viewed at most angles, a positive image of the scene is seen as areas of amalgam (diffusely reflecting), which appear white, on areas of polished silver (specularly reflecting), which appear black. The sharpness with which edges were reproduced in several old daguerreotypes led us to suspect that electrostatic processes might be involved, and we now report two experiments which confirm that.

To demonstrate that mercury vapour in air contains electrically charged droplets, we oxidized a single crystal silicon wafer, 10 cm in diameter, to form a surface film of silicon dioxide about 1-μm thick. A patterned stainless-steel mask (Buckbee-Mears Co., St. Paul, Minnesota) was placed on the oxidized surface, and the open spaces were charged with air ions from a point corona discharge (using a 'Zerostat'⁵ antistatic pistol). Such charge patterns can be rendered visible by immersion in liquid toners, as used in office copying and electrographic printing machines, which consist of electrically charged particles of colloidal carbon of 100-nm diameter suspended in an insulating liquid⁶. Here, the wafer bearing the charge image was placed over a beaker of mercury heated to about 70 °C. When the image was negatively charged, it gradually became visible as droplets of mercury condensed on it (Fig. 1). This experiment clearly demonstrates that positively charged droplets must form in the space between the mercury surface and the wafer, and that they

are attracted to negatively charged areas of the electrostatic image. The fact that some fraction of all finely divided particles and droplets is usually electrically charged is, of course, well known (for example, see Ref. 7).

To demonstrate that those areas of a daguerreotype plate exposed to light do indeed become negatively charged, we prepared some daguerreotype surfaces by sputtering silver on glass plates and exposing them to iodine vapour at room temperature (about 25 °C) for periods of up to an hour in darkness or in red light. The stainless-steel mask now clamped to the surface of the plate was then exposed to direct sunlight for about a second. The exposed plate, after removing the mask, was immersed in liquid toner containing positively charged particles, whereupon black toner particles were immediately deposited uniformly on the exposed parts of the surface. After rinsing in clear carrier fluid and drying, a clear image of black on silver was obtained (Fig. 2). We also prepared scanning electron micrographs to confirm that toner particles were indeed clustered on the exposed areas (Fig. 3). Such liquid-toned images on daguerreotype surfaces could at first be smeared or transferred to adhesive tape. Fixing was accomplished by heating, as the toner also contains a wax for this purpose. The silver amalgam image prepared by the original Daguerre process can also be smeared and transferred easily, and it remains so even after 'fixing' by heating to about 60 °C. These observations suggest that a photographic transfer process could possibly be based on easily prepared Daguerre surfaces.

An alternative explanation is that the surface becomes positively charged during exposure to iodine vapour and that the charge is dissipated in those areas exposed to light. This hypothesis was shown to be invalid, because we found that exposed plates produced no visible image at all when using negatively-charged toners. Furthermore, the deposition of positively-charged toner in the light exposed areas is very uniform. If the area were uncharged, development would occur only around the edges of the uncharged areas⁷. This effect also explains why negatively-charged toner does not give a visible image.

Xerographic layers can typically withstand electric fields due to surface charge of 10-30 V μm⁻¹ (ref. 7); thus we expect the surface potential of a 30-nm thick silver iodide layer to be in the range 300-900 mV. However, we were unable, using a

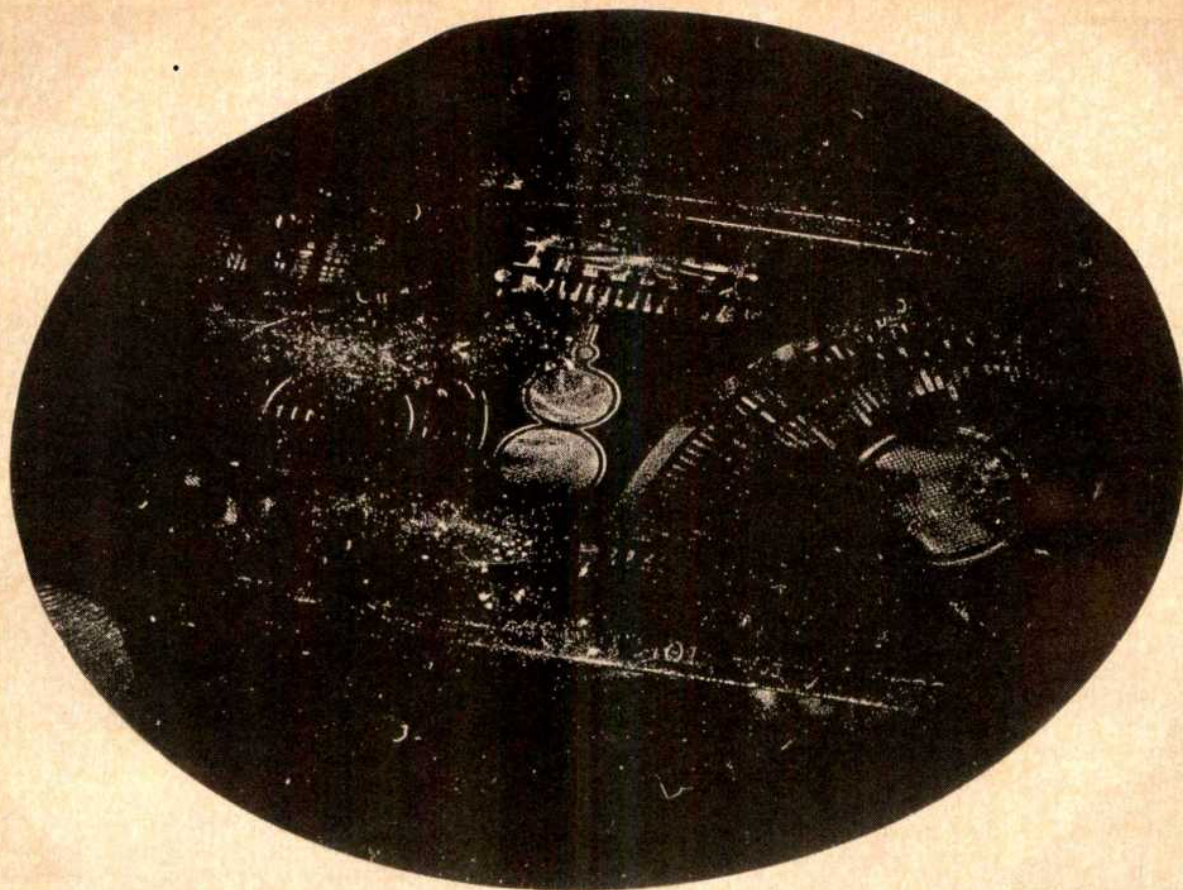


Fig. 1 Image—negatively charged by air ions from a point corona discharge—formed on a surface of oxidized silicon suspended in condensing Hg vapour.

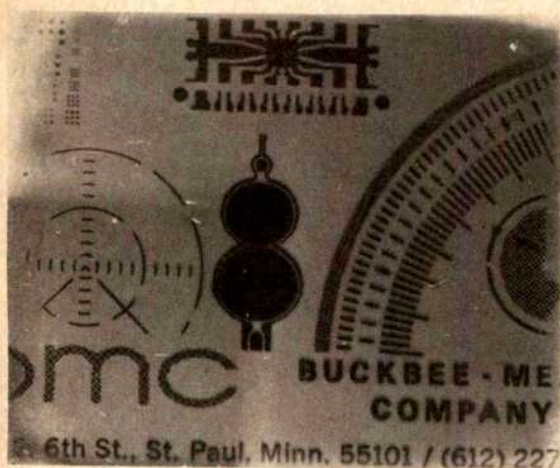


Fig. 2 Image of black toner particles on a surface of iodized silver. The AgI-coated plate, after exposure to direct sunlight, is immersed in liquid toner containing charged particles (Savin Corporation, Stamford, Connecticut).

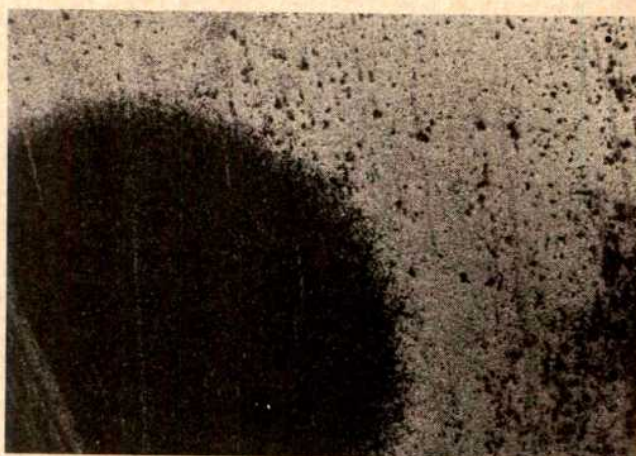


Fig. 3 Scanning electron micrograph of carbon toner on silver iodide.

Monroe electrostatic voltmeter (J. Monroe Electronics Inc., Lyndonville, New York) to detect any effects, due to charge, on the exposed silver iodide films. This was probably because the probe could not be brought any closer than about 100 μm from the surface, and the instrument is not sensitive enough at this distance to detect such low voltages.

It is interesting to speculate about the physical mechanism of the observed optical charging. As is well known, in the normal photographic process with silver halides, exposure to light causes a latent image of metallic silver atoms to be formed and the release of bromine atoms. The bromine atoms are highly unstable, and recombination to silver bromide (fading of the

latent image) occurs rapidly in the absence of a gelatin matrix⁸. In the case of daguerreotype plates, it is possible that the high electron affinity of iodine atoms may be satisfied by diffusion of electrons from the nearby substrate of metallic silver, thus contributing to the negative charge image. Alternatively, photoelectrons ejected from the silver interface may become trapped in the overlying silver iodide layer. Either process may explain the increased sensitivity obtained by adding to the iodine the halogens of greater electron affinity.

Further research may be able to discriminate between these and other possible mechanisms for the photocharging behaviour observed with daguerreotype plates. The use of liquid toners

with positively-charged colloidal particles of white coloration (such as titanium dioxide) will produce daguerreotypes without resorting to the toxic mercury vapour process.

Received 17 July; accepted 17 October 1984.

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Relaxation of stored mechanical stress along chemical reaction pathways

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Mechanical stress induced in a condensed material relaxes by way of viscous flow, that is by slippage of neighbouring molecules past each other and by the frictional dissipation into heat of the potential energy between them. We show here that this is not necessarily the only way by which energy of deformation can be dissipated. The breaking of chemical bonds within mechanically stressed molecules participating in a chemical reaction provides an alternative and parallel pathway for relaxation and is thus a factor in rheological description which has not been adequately recognized. A chemical change not only alters the composition of the system, and thus the response of its material, but also directly modifies the time scale of the relaxation modes of the molecules participating in the reaction. The involvement of chemical reactions in rheological processes has very broad implications, as most real systems—certainly all living systems—undergo chemical changes, although the rates of these may vary considerably.

Stresses and strains are induced when a force is applied to the surface of a condensed material system. In the resultant deformation, the atoms of which the material is composed are displaced from the potential energy minima characterizing the unstressed state. The internal stresses so generated must balance at each point. Hence, the extent of the displacement of the various atoms depends on the nature and steepness of the local potential energy gradient. For example, a very small displacement will generate the required stress between atoms linked covalently to each other, whereas a much larger shift in position will be needed to generate an equal force between two non-covalently linked first-neighbour atoms. Superimposed on these deterministic, internal, stress-producing interatomic shifts are spontaneous, random, thermal displacements in the material. These are always present, at a finite absolute temperature, even in the stress-free case, and in thermodynamic equilibrium. They involve not only the rapid vibrations of individual atoms about their potential energy minima, but also the slower transitions of assemblies of atoms, that is, of groups, molecules or complexes, from minimum to minimum and back. When stresses generated by externally applied forces are present, they act to bias these transitions and thus to produce permanent deformation or flow. Different materials are distinguished from each other by the number and nature of the groups, molecules or complexes which are capable of substantial change in form or position at the temperature in question within the time scale of interest.

It is often possible to describe a material system as a linear combination of mechanisms for the relaxation of applied stress, with each mechanism corresponding to a degree of freedom of the atoms of which the system is composed, and associated with a time whose magnitude depends on the primary and secondary

structure of the molecules of the system and their relationship to surrounding molecules. Each mechanism makes a contribution of order $k_B T$ to the modulus for instantaneous deformation, where k_B is the Boltzmann constant and T is the absolute temperature. The instantaneous modulus, that is, the stress induced per unit volume by unit strain instantly applied, is thus determined by the number of mechanisms per unit volume. As the number of mechanisms is a measure of the number of degrees of freedom, that is, of the number of atoms per unit volume, the instantaneous moduli of condensed phases are all relatively similar. The differences between material systems arise from the ways in which these mechanisms, essentially constant in total number per unit volume, are distributed over the relaxation-time axis, that is, by the spectrum of mechanical relaxation times.

When deformations are small, or flow is sufficiently slow, all events are linearly additive. It has then been usual to assume that, despite deformation, relaxation and flow, the material remains chemically and rheologically the same at all times. There are neither mechanically-induced chemical changes, nor chemically-generated compositional changes superimposed on the mechanical workings of the system.

The question may, however, be asked, what are the effects on the mechanical relaxation of such a system if its molecules can interconvert chemically¹? These effects, it turns out, transcend the obvious ones resulting from the change in composition². The molecular rearrangements caused by the chemical reaction provide additional pathways along which energy of deformation, stored in the mechanical relaxation mechanisms of the reacting species, can be transferred or dissipated into heat. The instant 'switching on' of a chemical reaction thus has two consequences: (1) the relaxation times characteristic of the mechanical relaxation pathways of the reacting molecules in the absence of chemical reactions are instantly lowered, and (2) the composition of the system and hence the number of mechanisms corresponding to each relaxation time is altered at the rate of the chemical reaction^{1,3,4}.

Therefore, if the change in mechanical response characteristics, during the period of chemical transition, is measured by the dynamic storage and loss moduli, the transients in these parameters should show the effects of the changes, both in the relaxation spectrum and in composition³. Hence, a homogeneous system, already possessing the composition of the reaction in chemical equilibrium and thus not subject to compositional change, will nevertheless produce a transient when the reaction, originally 'frozen', is initiated. In the general case of arbitrary starting composition, of course, the consequences of both effects are experienced.

In order to exemplify these ideas, let us consider a system composed of three rheologically-active macromolecular species which can interconvert. The chemical reactions will be assumed to alter the stiffness of the polymeric chains. For example, let us assume that there is a ring system associated with each monomer, which reduces the flexibility of the chain. Let us further assume that there are catalysts present which can selectively open or close these rings. Let species 1 be the stiffest polymeric species and let each molecule of 1, in dilute solution, contribute a Rouse-like relaxation time spectrum, $\tau_{1(1)} = 1$, $\tau_{1(2)} = 1/4$, $\tau_{1(3)} = 1/9$, ... $\tau_{1(p)} = 1/p^2$... We assume that there are four species of catalysts present: one of these, (21), converts a molecule of 1 into a molecule of 2 by opening the ring structure at every second monomer unit; another, (32), reacts with a molecule of 2 and opens all of the remaining ring structures, converting it into a molecule of 3; another, (23), reverses the action of (32); and the remaining one, (12), reverses the effect of (21). Catalysts (21) and (32), acting simultaneously, take a molecule of 1 directly into one of 3, and catalysts (23) and (12), acting together, cause 3 to revert to 1. Molecules of 3 are thus more flexible than those of 2 and the respective Rouse relaxation time spectra of species 2 and 3 are taken to be $\tau_{2(1)} = 1/9$, $\tau_{2(2)} = 1/36$, $\tau_{2(3)} = 1/81$, ... $\tau_{2(p)} = 1/(3p)^2$, ... and $\tau_{3(1)} = 1/100$, $\tau_{3(2)} = 1/400$, $\tau_{3(3)} = 1/900$, ... $\tau_{3(p)} = 1/(10p)^2$, ... In practice, we shall consider only the first three relaxation mechanisms in

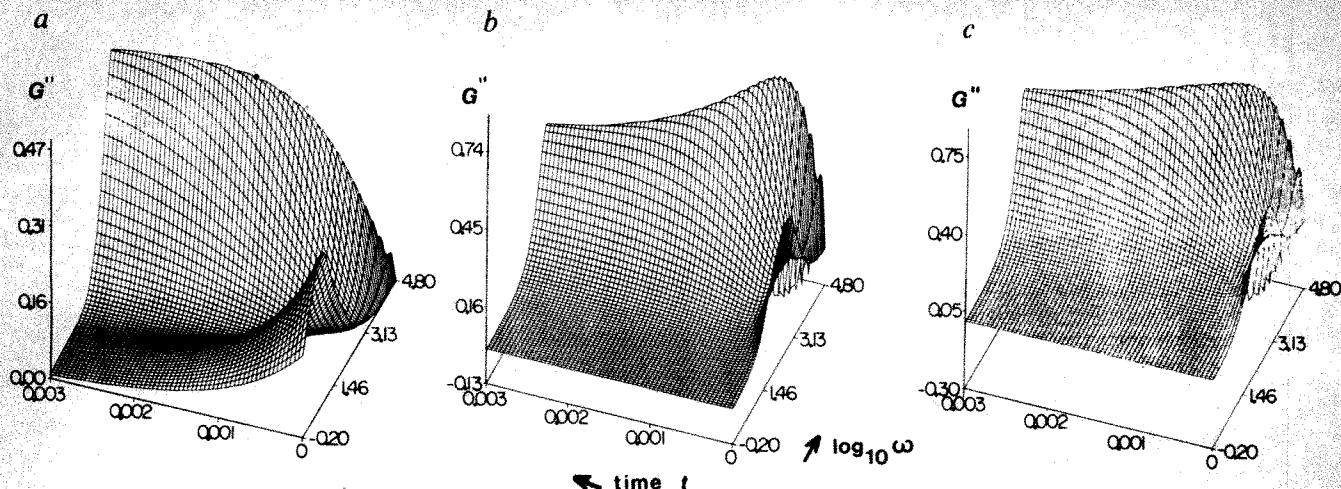


Fig. 1 Frequency (ω) and time (t) dependence of the dynamic shear loss modulus (G'') in the presence of a chemical reaction transient. Units are arbitrary, but the same for *a*, *b* and *c*. The system comprises three macromolecular species, possessing truncated Rouse relaxation-time spectra, undergoing macromolecular interconversions; only the three longest relaxation times are considered in each case. Oscillations started at time $t = -100$. The chemical reaction started at time $t = 0$. The initial composition in *a* matches the composition at chemical equilibrium; in *b* and *c* the initial concentrations of species 1 and 2 are zero. The dissipation modes are: *a*, *b*, no transfer of energy of deformation from reagents to products and total direct dissipation into heat as each stressed molecule reacts; *c*, partial transfer of energy of deformation from reagents to products with the remaining energy dissipated directly into heat. Note that the experimental determination of G'' , at a certain frequency ω , requires a minimum of time of the order $1/\omega$. During this period, the system should remain essentially unchanged chemically. Otherwise, the measurement becomes difficult to interpret. If the mean chemical conversion time is $1/\langle k \rangle$, then the time for measurement, $1/\omega$, must be shorter. Hence only that portion of the theoretical transient in G'' lying at frequencies ω larger than $\langle k \rangle$ can conveniently be verified. In the present example, the chemical transient occurs between $t = 0.0001$ and 0.001 , so that the critical value for $\log_{10} \omega$ lies between 3 and 4.

each case. The time scale is arbitrary and is taken with respect to the longest relaxation time, $\tau_{i(1)}$, which is set equal to one. The three species interconvert according to the following reaction scheme

$$\frac{dn_i}{dt} = \sum_{j=1}^3 k_{ij} n_j; \quad i = 1, 2, 3 \quad (1)$$

where the n_i are the concentrations; the k_{ij} are the rate constants for the conversion of j into i , if $j \neq i$, and k_{ii} is the rate at which i reacts to form the other two species. Thus $k_{ij} \geq 0$ if $j \neq i$, whereas $k_{ii} < 0$. The time t and the k s are on the same reduced time scale as the τ s. The concentrations of the four catalysts described, [12], [23], [32] and [21], stay constant and are included in the appropriate constants k_{ij} . For example, $k_{12} = k'_{12}[12]$, $k_{11} = -k'_{21}[21] - k'_{31}[32][21]$, etc. In our case, let the scheme K of rate constants have the following values

$$K = (k_{ij}) = \begin{pmatrix} -1 & 2 & 5 \\ 2/3 & -3 & 5 \\ 1/3 & 1 & -10 \end{pmatrix} \times 10^3 \quad (2)$$

The solution of equations (1) and (2) implies that, at equilibrium, the concentrations n_i^* of the three species are in the following ratio:

$$n_1^* : n_2^* : n_3^* = 15 : 5 : 1 \quad (3)$$

If we now assume that each macromolecule has the same number of mechanisms $\hat{H}$ associated with each of its relaxation times, then the number of mechanisms will be given by $N_{i(p)} = \hat{H} n_i$. Consequently, the number of mechanisms per unit volume, at equilibrium, $N_{i(p)}^*$, will also be in the ratio given by equation (3).

If linear viscoelastic behaviour is assumed and there is no chemical reaction, the stress $\sigma_{i(p)}$ induced in the $N_{i(p)}$ mechanisms by a time rate of strain $d\gamma/dt$ will be given by

$$((d/dt)I + \Lambda)\sigma = k_B T N (d\gamma/dt) \quad (4)$$

where σ and N are vectors having the nine quantities $\sigma_{i(p)}$ and $N_{i(p)}$, respectively, as elements. In equation (4), I is the (9×9) identity matrix, and Λ is a (9×9) diagonal matrix with the nine quantities $1/\tau_{i(p)}$ along the diagonal. The integration of equation (4) is straightforward.

Let the system have a composition $N = N^0$ at the time, $t = 0$, when the reaction (1) is started. With the start of the reaction, N , in addition to σ and $d\gamma/dt$, becomes a function of time. If this were the only effect of the reaction, the principle for solving equation (4) would not be changed. However, when a molecule of j reacts to form a molecule of i , the energy of deformation stored in the mechanisms $j(s)$ of that molecule must either be transferred to the mechanisms $i(p)$ of the molecule i , which is formed at the rate of the chemical reaction, or be totally or partially dissipated as heat.

To allow for this, we introduce the stored-stress rate of distribution matrix, A . This is a (9×9) matrix whose elements are given by

$$A_{i(p)j(s)} = \alpha_{i(p)j(s)} k_{ij} \quad (5)$$

where $\alpha_{i(p)j(s)}$ is the fraction of the stress stored in a mechanism $j(s)$ which is transferred to the mechanism $i(p)$ of the molecule i as it is formed. Clearly $\sum_p \alpha_{i(p)j(s)} \leq 1$ and $(1 - \sum_p \alpha_{i(p)j(s)})$ represents that fraction of the stress stored in the mechanisms $j(s)$ at time t which is dissipated as heat. At times $t > 0$, equation (4) must be replaced by

$$((d/dt)I + \Lambda - A)\sigma = k_B T N (d\gamma/dt) \quad (6)$$

If we assume that all of the stored energy of deformation is dissipated as heat as the molecules react, the off-diagonal elements of A are all zero. In this case, equation (6) can be written

$$((d/dt)I + \Lambda')\sigma = k_B T N (d\gamma/dt) \quad (7)$$

where Λ' is a diagonal matrix with elements $1/\tau'_{i(p)}$ on the diagonal, given by

$$1/\tau'_{i(p)} = 1/\tau_{i(p)} - k_{ii} \quad (8)$$

As the k_{ii} in equation (8) are negative, $\tau'_{i(p)} \leq \tau_{i(p)}$. The integration of equation (7) is again straightforward.

If γ is taken to be a sinusoidally-varying shear strain of constant amplitude γ_0 and frequency ω , we can compute the real and imaginary components, $G'(\omega)$ and $G''(\omega)$ respectively, of the complex dynamic shear modulus³. Because of the reaction, both $G'(\omega)$ and $G''(\omega)$ become functions of time, at $t \geq 0$, until chemical equilibrium is established. Frequency, ω , is again reduced to the same time scale as τ .

Figure 1 illustrates what happens in the cases discussed above: in Fig. 1a, the initial mechanism concentrations N^0 are equal to those at chemical equilibrium, N^* ; in Fig. 1b and c, the initial mechanism concentrations are $N_{1(p)}^0 = N_{2(p)}^0 = 0$ and $N_{3(p)}^0 = \text{constant}$. Thus, changes in composition when the reaction is switched on occur only in the latter two cases. Each curve in the $t = \text{constant}$ plane gives G'' as a function of the frequency ω ; these curves are zero-order approximations to the relaxation time spectrum, with $1/\omega$ to be regarded as a measure of τ . The curve at $t = 0$ is thus the spectrum characterizing the system at all times before the start of the reaction.

Because, in the case in Fig. 1a, there is no change in N , the transient observed is owing to a switch from the spectrum $\tau_{i(p)}$ to the spectrum $\tau'_{i(p)}$ (equation (7)). The alternative pathway for dissipation of energy of deformation that is suddenly opened through the reaction accounts for the transient in G'' . However, in the case in Fig. 1b, N also changes at times $t \geq 0$. The transient is thus different, but the final states in Fig. 1a, b and c are all the same. Figure 1c illustrates the case of a non-diagonal A , that is, a case where there is also a transfer of energy of deformation, from mechanism to mechanism, as the macromolecules interconvert. The difference between Fig. 1b and c is very small, however, and this seems to be typical of all cases of non-diagonal A s which we have examined.

We have chosen the relaxation times $\tau_{i(p)}$ of each macromolecular species to follow the pattern of a typical Rouse spectrum, but have used in our computations only the first three relaxation modes ($p = 1, 2, 3$) at the long-time end of the spectrum. This accounts for the unusual frequency dependence of G'' , which lacks its usual high-frequency part.

The process by which displacement-induced stresses relax can be viewed as a progressive loss of interatomic correlation. At any instant, a system will be in a definite configuration and all of its component atoms can be identified and assigned definite position coordinates. At that moment there is total correlation, but as time progresses, most of these correlations are lost. Flexible molecules will deform, but correlations between atoms in covalently linked molecules will, on the whole, be maintained. Correlations are lost as a result of two processes: spontaneous thermal agitations and chemical reactions. The effectiveness of thermal agitation depends on the organization of the secondary bonds between the molecules in the system. Systems can range from being almost always perfectly correlated in their secondary bond structure, as in crystals at low temperature, to those suffering an almost immediate loss of identity, as in gases at high temperature. When a chemical reaction is superimposed, some covalent bonds can now break and re-form, as well. As a result, certain additional correlations are no longer maintained and a further pathway for configurational rearrangements, that is, for material displacement and flow, has been gained.

An example will illustrate these ideas. Let AA react with BB to give 2AB. The correlation between the atoms in each of the parts A is maintained, but not those between the two parts of molecule AA, although A does not occur as an independent molecular species. In such a system, relaxation of mechanical stress can take advantage both of the capacity of a grouping A to undergo thermal displacement as part of a molecule of AA, and of the capacity of this grouping to transfer, in a chemical reaction, from AA to another AA or to AB. There is a sense, therefore, in which it is more appropriate to view such a system of molecules AA, BB and AB as a system containing only the entities of maintained correlation, A and B. Mechanical stress relaxes by taking advantage of all mechanisms by which arrangements of entities such as A and B become decorrelated.

We have considered in this analysis only the fate of energy of deformation stored in molecules subject to the 'trauma' of a chemical reaction. During the energetic upheaval involved in such a chemical reaction, we have supposed that all, or part, of the stored mechanical energy is dissipated into heat at the rate of the reaction. We have not considered here the effect that the energy put into the molecules mechanically may have upon the rate of the reaction and its equilibrium, say, by favouring either

the forward or the reverse process (thixotropy or anti-thixotropy)⁵. We have also not considered here the possibility that some of the energy released by the reaction could find its way into mechanical deformation modes and thus produce changes in shape and motion directly, that is, by acting as a converter of chemical energy into mechanical energy via immediate molecular pathways (mechanochemistry⁶), rather than through specialized macroscopic devices such as engines and motors.

Received 12 July; accepted 17 October 1984.

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Ruthenium in the ocean

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Ruthenium, one of the rarest metals of the platinum group, has not previously been investigated in ocean waters and sediments because of its low concentration therein and the lack of sensitive analytical methods. However, the study of ruthenium distribution in the ocean is of interest not only in oceanology but also in geology, geochemistry and cosmochemistry, as it may help in understanding the lithosphere formation processes. We report here the results of ruthenium trace determination in various oceanic environments carried out using a combination of methods including fire assay enrichment and laser photoionization spectroscopy in conjunction with vacuum atomization¹. The ruthenium content in the marine environment fluctuates in the range covering 4-5 orders of magnitude: ~ 1 p.p.t. (parts per 10^{12}) in marine water, tens of p.p.t. in marine biogenic material, 10-100 p.p.t. in bottom sediments and marine phosphorites, and $>1,000$ p.p.t. in ferromanganese nodules and the Red Sea metalliferous sediments.

As the analytical information on ruthenium is limited, its abundance in the Earth's crust has only roughly been estimated at 10-100 p.p.t. (ref. 2). Using conventional analytical methods for determining the Ru content in natural objects, the best results (detection limits 1-10 p.p.b. (parts per 10^9))³ have been obtained only after preliminary chemical concentration⁴ or fire assay concentration⁵ of ruthenium from large (100-500 g) samples. The complexity of the multistage chemical treatment of samples and the volatility of ruthenium oxides make such a concentration technique labour-consuming and not very reliable. The method of fire assay concentration of ruthenium in lead alloy is more convenient, and this is what we have used in our experiments.

The fire assay enrichment procedure^{5,6} consisted of fusing 25 g of the sample with litharge (PbO) and appropriate fluxes at 1,000 °C. Ruthenium is collected in a metallic lead alloy (lead button) of mass 30-40 g. After mechanical separation of slags, the lead button produced was reduced to 50-100 mg by oxidizing in a porous vessel (cupel) with an air flow at a temperature of 900-1,000 °C. According to Kolosova⁵ such lead button reduction does not cause ruthenium losses. When analysing seawater, the volume of about 1 l of acidified water was evaporated to dryness, followed by fire assay enrichment of salt. The enrichment factor obtained by this method was 10^2 - 10^4 .

Table 1 Chemical composition and Ru content of investigated samples

Sample	Location [lat., long., depth (m)]	Ru* (p.p.t.)	Fe ₂ O ₃ (%)	MnO (%)	Al ₂ O ₃ (%)	P ₂ O ₅ (%)	Ni (%)	Cu (%)	Co (%)
1. Clayey-siliceous ooze	Northern Pacific [14°23' N, 117°18' W, 4,125]	250	3.25	0.50	11.09	ND	0.019	0.0037	0.0042
2. Clayey-siliceous ooze	Southern Indian Ocean [8°45' S, 102°19' E, 5,300]	150	4.2	1.1	9.5	ND	0.024	0.022	0.0028
3. Metalliferous sediment	East Pacific Rise, [38°56' S, 99°58' W, 4,330]	1,700	26.32	8.30	8.36	ND	0.097	0.12	0.0228
4. Metalliferous sediment	Red Sea, Discovery Deep	7,600	10.5	0.30	0.6	ND	0.001	0.10	0.010
5. Ferromanganese nodule	Northern Pacific, [14°23' N, 117°18' W, 4,125]	3,200	7.93	38.17	4.68	0.21	1.12	0.97	0.15
6. Ferromanganese nodule	Northern Pacific, [10°58' N, 153°22' W, 4,980]	3,500	9.15	37.80	5.05	ND	1.23	1.05	0.24
7. Ferromanganese nodule	Southern Pacific, [22°45' S, 160°50' W, 4,850]	1,600	24.70	22.60	5.95	ND	0.34	0.20	0.40
8. Ferromanganese crust	Mid-Pacific Mountains [20°41' N, 170°32' W, 1,930]	3,800	23.80	29.50	2.33	ND	0.35	0.08	0.65
9. Phosphorite (Pliocene)	Sea of Japan [38°43' N, 130°12' E]	23	2.41	0.04	1.44	27.98	0.002	0.001	0.001
10. Phosphorite (Pleistocene)	Mid-Pacific Mountains, [32°16' N, 172°51' E, 370]	170	0.50	0.10	0.20	16.7	0.001	0.001	0.001
11. Phosphorite (Cretaceous)	Mid-Pacific Mountains, [18°31' N, 175°06' W, 1,050]	260	0.38	0.10	0.51	27.9	0.006	0.004	0.001
12. Phosphorite (Holocene)	Namibian shelf, [22°08' S, 13°58' E, 85]	660	0.30	0.001	Traces	31.5	0.0016	0.0003	0.0001
13. Fish bones	Chile shelf, [21°07' S, 70°21' W, 150]	56	1.5	0.02	Traces	29.5	0.0001	0.002	0.0001
14. Whale vertebra	Namibian shelf, [17°24' S, 11°33' E, 120]	90	0.2	0.01	0.25	27.8	0.0003	0.003	0.0001
15. Whale rib	Namibian shelf, [19°24' S, 12°15' E, 130]	350	1.6	0.01	Traces	31.1	0.0005	0.001	0.0001
16. Ocean water	Bay of Biscay, Surface	1.3	70†	40†	1,000†	70,000†	500†	250†	1†
17. Interstitial water of siliceous-clayey ooze	Southern Indian Ocean, [8°45' S, 102°19' E, 5,300]	6.9	—	—	—	—	—	—	—
18. Atmosphere aerosol	Northern Atlantic, [20 March 1983, 5°43' N, 29°13' W– 21 March 1983, 10°37' N, 27°59' W]	910	—	—	—	—	—	—	—

ND, Not determined.

* Ruthenium values are averages of triplicate analyses and analytical precisions (1σ) are 15 and 25% for solid and water samples respectively.

† After ref. 15 in p.p.t.

The lead produced after cupellation was put into the graphite crucible of the electrothermal atomizer inside a vacuum chamber. After it was evacuated to a pressure of 10^{-6} mm Hg, the crucible was heated to 900 °C. In this case lead evaporated, while ruthenium, because of its extremely low volatility, was concentrated in the residuum. In this way, the matrix was evaporated for 15–40 min. The rest of the sample was atomized with the crucible temperature increasing gradually up to 2,400 °C. The resulting atomic vapour was formed by the cylindrical channel of the crucible into an atomic beam. This beam was irradiated between two plane electrodes by the radiation of three pulsed dye lasers tuned for three-step excitation of ruthenium atoms to the Rydberg state $4d^7 5s(^5F_5) \xrightarrow{392.6 \text{ nm}} 4d^6 5s 5p(^7D_2) \xrightarrow{633.05 \text{ nm}} 4d^7 6s(^5F_5) \xrightarrow{567.33 \text{ nm}} 4d^7 17p$. 20 ns after laser excitation the Rydberg atoms of ruthenium were ionized by an electric field pulse fed to the electrodes. The ions passed through the slit in one of the electrodes and were detected by an electron multiplier. The laser photoionization analytical spectrometer is described in detail in ref. 1.

Figure 1a shows the typical dependence of an ion signal on evaporation time with the atomizer temperature increasing gradually. The content of ruthenium in the sample is determined by integral selective ion signal. To distinguish the Ru selective signal from the total ion signal, the frequency of one laser was tuned off the resonance of the corresponding transition⁷. The ion signal was calibrated using a curve constructed on the basis of copper samples containing a standard quantity of ruthenium. The possibility of such calibration was confirmed by the equality of the signals from copper and lead alloys which had collected ruthenium from standard samples of ruthenium-bearing ore. The detection limit of Ru in a lead bead was about 0.5 p.p.b.

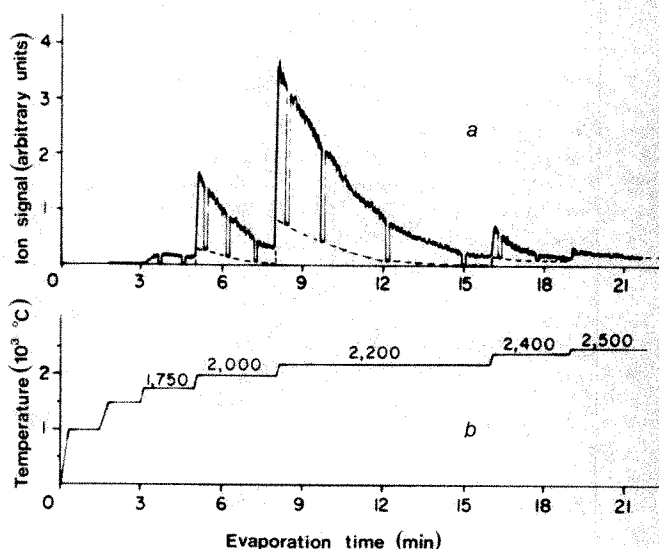


Fig. 1 a, Typical dependence of the ion signal on evaporation time and atomizer temperature for the lead bead of 100-mg weight. The dips are caused by the detuning of the first-step dye laser wavelength from the Ru absorption line to provide a reference baseline. The selective signal of ruthenium is defined by the area between the ion signal curve and the dashed line. b, Time dependence of the atomizer temperature.

Taking into consideration the concentration factor, the Ru detection limits are 1 p.p.t. for solid samples and 0.03 p.p.t. for waters. Typical blanks ranged from less than the detection limit to 7 p.p.t. (solid samples) and 0.2 p.p.t. (waters).

Table 1 lists the investigated materials and the results obtained. The ruthenium content in the biogenic siliceous-clayey sediments from the Pacific and Indian Oceans amounts to 150–250 p.p.t. In the metalliferous sediments enriched in iron and other metals, the ruthenium content is much higher: in the sample from the East Pacific Rise, 1,700 p.p.t.; from the Discovery Deep in the Red Sea, 7,600 p.p.t. Both samples show 7–8-fold enrichment in iron as well as in ruthenium, compared with ordinary sediments. The comparison with other metals listed in Table 1 shows no such correlation.

The metalliferous sediments of the Red Sea are characterized by the high content of several metals, including gold and silver⁸, which could be explained by the corrosive nature of brines percolating rocks and sediments of the rift zone on their way to depressions. Thus, the ore fluids can be enriched with heavy metals in different ratios depending on the local conditions.

Another group of ocean formations enriched in iron, manganese and heavy metals are ferromanganese nodules and crusts⁹. The ruthenium content in investigated nodules varies between 1,600 and 3,500 p.p.t. The lowest content is found in the nodule from the South Pacific. Whereas two samples from the North Pacific show the two-fold enrichment. The maximum of 3,800 p.p.t. is found in the ore crust from a sub-sea mountain of the northern part of the ocean. Comparing these results with the other metal contents shows that the distribution of ruthenium might be correlated with nickel variations in the nodules and cobalt variations in the crust. The high content of ruthenium had been previously determined in standard nodules from Pacific (4,700 p.p.t.) and Atlantic nodules (18,000 p.p.t.)¹⁰. Various kinds of ultramafic rocks contain from 700 to 2,000 p.p.t. of Ru on average¹¹, commensurate with the Ru content in ferromanganese nodules, but lower than that in the metalliferous sediments occurring in the Red Sea.

Phosphate rocks consisting mainly of calcium phosphate belong, too, to the genetically-complex formations on the sea floor¹². In the samples that we studied, ruthenium content varies between 23 and 660 p.p.t. The phosphorite sample from the sub-sea mountain in the Sea of Japan is the lowest in ruthenium containing only 23 p.p.t. In the phosphate rocks from the mid-pacific mountains, the content of ruthenium is much higher (170–260 p.p.t.) and reaches its maximum in the lithified Holocene nodule from the inner shelf of Namibia, 660 p.p.t. The distribution of ruthenium in the analysed phosphorites does not correlate with any of their macro- or microcomponents. The only specific feature of chemical composition of the phosphorite that differentiates it from the other ones is an increased content of organic matter in it¹², which might be the cause of ruthenium concentration.

In the fish and marine mammal bones from the bottom of the shelf regions of the ocean, the ruthenium content ranges from 56 to 350 p.p.t. In the slightly lithified fish bones from the shelf of Chile it reaches 56 p.p.t. and increases in the slightly lithified porous whale vertebrae and rib from the shelf of Namibia to 90 and 350 p.p.t. The behaviour of ruthenium in this case seems to depend on nickel, its content increasing from 0.0001 to 0.0005% respectively.

In the sample of seawater from the Bay of Biscay, ruthenium makes up 1.3 p.p.t. This value is almost the same as the ruthenium content cited in ref. 13 but two orders of magnitude higher than that of palladium determined by Lee¹⁴. Thus, the concentration of ruthenium in seawater is two orders of magnitude lower than in pelagic sediments. Most other heavy metals are characterized by similar feature: their content in seawater is usually 3 or 4 orders lower than in sediments¹⁵.

In the interstitial water of siliceous-clayey ooze from the Indian Ocean, ruthenium content is 6.9 p.p.t. This shows that in the process of pelagic ocean diagenesis, ruthenium is able only to undergo some redistribution between the solid and liquid

phases of sediments. The enrichment of ferromanganese nodules with ruthenium may depend on both its supply from seawater and interstitial water.

Of particular interest is a high content of ruthenium in the atmosphere aerosol sample (910 p.p.t.) taken in the centre of the North Atlantic at the latitude of Sahara. This concentration is about three orders of magnitude higher than in seawater and 4–5 times higher than in pelagic bottom sediments. Such enrichment may be considered as evidence of cosmogenic supply of ruthenium to the ocean owing to high ruthenium content in cosmogenic materials^{16,17}. Similar hypotheses had been suggested for nickel and platinum metals concentrated in the oceanic sediments and in ferromanganese nodules^{18,19}.

Received 4 June; accepted 21 September 1984.

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A recently extinct palm from Easter Island

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The former existence of palms on Easter Island has been demonstrated palynologically^{1,2}, but the genus could not be determined from pollen morphology. We now report the discovery on the island of endocarps (shells) from palm fruits which appear to be those of an extinct species related to the Chilean 'wine palm', *Jubaea chilensis* (Molina) Baillon. The endocarps, found in caves, have all been gnawed by rodents, which could have helped to make the species extinct. The radiocarbon age of the endocarps is 820 ± 40 yr BP, which falls within the phase during which deforestation occurred on the island² and the giant statues (*moai*) were erected^{1,3}. Decline of the palm could have contributed to the decline of the *moai* culture.

The pollen record² shows that palms have existed on Easter Island since at least 37,000 BP, but declined to extinction within the past 1,000 years. The fossil pollen (Fig. 1a) is of the monocolpate type with a rough surface, which is common to many palm genera including *Cocos*⁴ and *Pritchardia*⁵, both widespread in the Pacific, and also *Jubaea* which occurs in Chile. The pollen was provisionally assigned to *Pritchardia* (O. Selling, quoted in ref. 1).

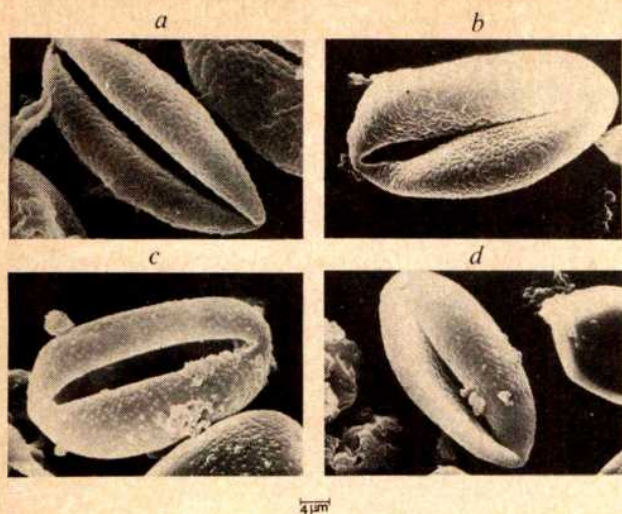


Fig. 1 Pollen grains in the scanning electron microscope. *a*, Fossil pollen from Easter Island; *b*, pollen of *Jubaea chilensis*; *c*, pollen of *Pritchardia minor*; *d*, pollen of *Pritchardia gaudichaudii*.

A few *Cocos nucifera* trees exist on the island today but these are of known introduction¹. Palm stumps, believed to be those of coconut, were seen in 1868 (ref. 6), but they could, presumably, have belonged to other species of palm. Legends describe the former existence on the island of a tree called *makoi nau opata* which had an edible fruit, and was used as food by the early settlers, but which no longer survives.

On 20 July 1983, J.-M. Groult and A. Gautier of the *Groupe d'Études et des Recherches Spéléologiques Rouen Ile de Paques* were exploring a newly-discovered cave near Ana Okeke on the cliffs of the Poike peninsula towards the eastern end of the island. On the floor of the cave they found about 30 palm endocarps (Fig. 2*a*), all gnawed. A further single endocarp, similar to the others, was found in August 1983 in a small recess in the face of Ahu Ma'itaki Te Moa on the north coast of the island by M. Symond.

Four of the best-preserved endocarps have been examined. The hard, 2–3-mm thick endocarps are faintly three-angled, three-edged and bear three clearly defined pores. They are clearly endocarps of a palm belonging to the cocosoid major group, and their identity can be narrowed down to the *Cocos* alliance⁷. Of the genera in this alliance, the Easter Island endocarps are closest to those of *Jubaea*, a monotypic genus of Chile, but are, however, consistently different from extant *J. chilensis* (Molina) Baillon (Fig. 2*b*). Three of the four endocarps available for examination are oblate, 30–34 mm in diameter at the equator; the fourth is subspherical, some 25 mm in diameter. The pores lie just below the equator. In *J. chilensis* the pores lie nearer to the base of the endocarp; the equatorial diameter does not exceed 24 mm. We conclude that the Easter Island endocarps belong to a cocosoid palm related to but distinct from *J. chilensis*. Unfortunately generic delimitation in this alliance is based largely on details of inflorescence and flowers; thus it is not possible to indicate whether the Easter Island palm is an extinct species of *Jubaea* or whether it represents a distinct genus.

We performed ¹⁴C dating on several of the fruits. Although the samples appeared to be well preserved, the soluble organic fractions, which may have represented postmortem contamination by foreign organic material, were removed by a series of alternating digestions in hot (80 °C) acid (2 M HCl) and alkali (0.5 M KOH). This decontamination procedure was followed by washing to neutral pH with distilled water. The residual carbon recorded a conventional radiocarbon age value (SRR-2430) of 820 ± 40 yr BP. Conversion of this radiometric age estimate via dendrochronological calibration⁸ places a 95% (2σ) confidence on the fruits having grown at some time in the calendar span AD 1040 to 1280.

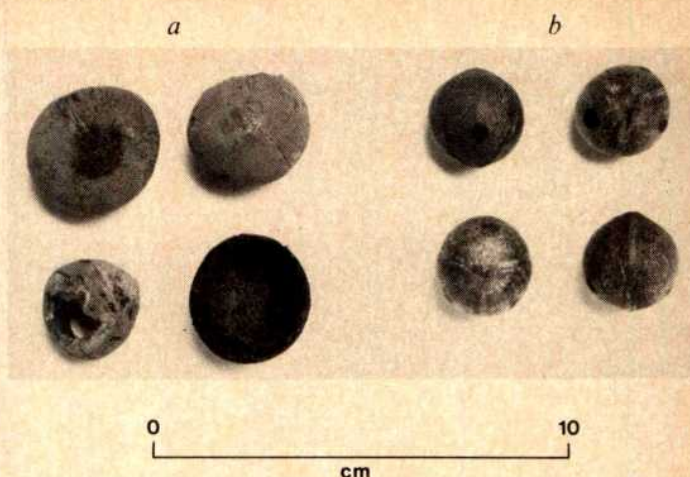


Fig. 2 *a*, Endocarps found in a cave on Easter Island. *b*, Endocarps of *Jubaea chilensis* cultivated in the Temperate House, Royal Botanic Gardens, Kew.

The ratio of stable carbon isotopes (¹³C/¹²C) in plant tissues can provide in many instances a clear indication of the metabolic characteristics of the species^{9,10}. Unfortunately in this respect the recorded enrichment value ($\delta^{13}\text{C}_{\text{PDB}} = -21.2\%$) falls firmly in the overlap between those ranges of ¹³C enrichment that can be recognized as indicative of the C₃ (Calvin cycle), C₄ ('dicarboxylic acid' cycle) or CAM (crassulacean acid metabolism) photosynthetic pathways.

The toothmarks on the endocarps are consistent with gnawing by rodents. The main rodent on the island today is the Norwegian rat, *Rattus norvegicus*, thought to have been introduced since Europeans discovered the island in AD 1722 (refs 1, 11). The Norwegian rat is believed to have ousted an earlier colony of Polynesian rats. In archaeological excavations at Anakena on the north-east coast of the island, we recovered bones of the Polynesian rat *Rattus concolor*, probably introduced by the early settlers. It is probable that this species gnawed the endocarps found in the cave, although the toothmarks are rather small to be those of rats (A. J. Stewart, personal communication). The same excavations produced numerous fragments of palm endocarps similar to those under discussion, suggesting that the fruits were also part of the human diet.

The pollen of *J. chilensis* (Fig. 1*b*) is similar to the fossil pollen (Fig. 1*a*) found in swamps on the island. Both pollen grains have a rugulate surface. The fossil pollen differs, however, from that of the two species of *Pritchardia* with which it has been compared (Fig. 1*c, d*). Both *Pritchardia* species have pollen grains with a fossulate, not rugulate surface.

From the pollen record², it appears that the extinct palm was important in the island vegetation; it may have been the dominant tree in the lowlands. Deforestation occurred in the past 1,000 yr (ref. 2); most of the giant statues were erected during the same period^{1,3}. Man is believed to have reached the island about AD 400¹, so it is possible that the extinction of the Easter Island palm was caused by a combination of direct deforestation and prevention of reproduction, the latter resulting from eating of the fruits by man and the introduced rats.

Not all members of the *Cocos* alliance have large erect stems, but if the extinct palm was similar in morphology to *J. chilensis*, it would have made suitable levers for the movement of the *moai*; its smooth cylindrical trunk could have been cut into rollers for the same purpose. The construction of *moai* ceased suddenly in AD 1680 (ref. 1); this may have been caused by the extinction of the palm.

J.R.F. acknowledges the assistance of the Chilean government in visiting the island and the Easter Island Museum in permitting removal of specimens. The remaining palm endocarps are retained at the museum.

Received 31 August; accepted 22 October 1984.

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Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*

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The *per* locus of *Drosophila melanogaster* has a fundamental role in the construction or maintenance of a biological clock. Three classes of *per* mutations have been identified: *per*¹ mutants have circadian behavioural rhythms with a 29-h rather than a 24-h period, *per*^s mutants have short-period rhythms of 19 h, and *per*⁰ mutants have no detectable circadian rhythms^{1–4}. Each of these mutations has a corresponding influence on the 55-s periodicity of male courtship song⁵. Long- and short-period circadian rhythm phenotypes can also be obtained by altering the dosage of the wild-type gene⁴: for example, females carrying only one dose of this X-linked gene have circadian rhythms with periodicities about 1 h longer than those carrying two doses. In a previous report⁶, cloned DNA was used to localize several chromosomal rearrangement breakpoints that alter *per* locus function. The rearrangements all affected a 7-kilobase (kb) interval that encodes a 4.5-kb poly(A)⁺ RNA. We report here that when a 7.1-kb fragment from a *per*⁺ fly, including the sequences encoding the 4.5-kb transcript, is introduced into the genome of a *per*⁰ (arrhythmic) fly by P element-mediated transformation, circadian rhythmicity of behaviour such as eclosion and locomotor activity is restored. The transforming DNA complements *per* locus deletions and is transcribed, forming a single 4.5-kb poly(A)⁺ RNA comparable to that produced by wild-type flies.

The 7.1-kb *Hind*III fragment contained in phage ZW106 (represented by a wavy line in Fig. 1) was inserted into Carnegie 20 (ref. 7) to form plasmid pCPI (Fig. 1). A mixture of p π 25.1 (ref. 8), which contains a functional P-factor, and pCPI, which contains a defective P-factor that can be rescued by p π 25.1 and a wild-type *rosy* gene (*ry*⁺) that affects eye colour, was microinjected into the region of pole cell formation of preblastoderm *y per*⁰; *ry*⁴² embryos. Survivors were pair-mated with *y per*⁰; *ry*⁴² flies, and F₁ progeny were scored for wild-type eye colour, which indicates successful germ-line integration of pCPI sequences (see Fig. 1 legend). Transformed lines were maintained as heterozygotes, with respect to the transforming DNA, by backcrossing to *y per*⁰; *ry*⁴² flies.

One such transformed line, strain *per*⁰; P1.48C (*y per*⁰/Y; P1.48C/+; *ry*⁴²/*ry*⁴² males, and *y per*⁰/*y per*⁰; P1.48C/+; *ry*⁴²/*ry*⁴² females), carries a single transposon insertion at polytene chromosomal position 48C (chromosome 2R) by *in situ* hybridization. Hybridization of pCPI sequences to restriction digests of total genomic DNA from flies carrying P1.48C indicates that the transforming DNA is present in a single copy, and has the structure predicted from the restriction maps in Fig. 1 (data not shown). Derivatives of this strain of flies were subjected to a series of behavioural tests to determine whether

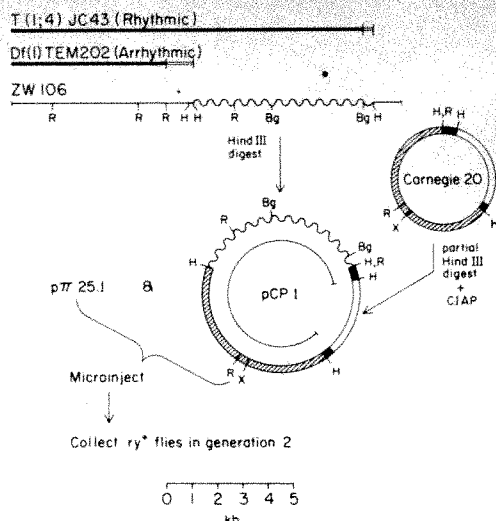


Fig. 1 Physical map of *per* region DNA and the construction of transforming DNA, pCPI. The positions of two chromosomal rearrangements, *T(1;4)JC43* and *Df(1)TEM202*, are shown relative to phage ZW106 (ref. 6). Solid bars represent DNA still present and in the wild-type configuration, while the location of each chromosomal rearrangement breakpoint is shown as an open bar⁶ (see text for details).

Methods: Transforming DNA was constructed as follows: ZW106 was digested to completion with the restriction endonuclease *Hind*III (H, *Hind*III; R, *Eco*RI; Bg, *Bgl*II; X, *Xho*I). The 7.1-kb fragment (shown as a wavy line in ZW106) was purified from a low-melting point agarose gel. Carnegie 20 (ref. 7) was partially digested with *Hind*III and treated with calf intestinal alkaline phosphatase (CIAP) to prevent self-religation. The 7.1-kb fragment was ligated to the partially digested vector and introduced into *Escherichia coli* (HB101). Transforming DNA sequences were mapped with restriction endonucleases and one, pCPI (shown in figure), was chosen for microinjection into *Drosophila*. pCPI contains the recombinogenic ends of a truncated P-factor⁷ (■), *per* locus DNA (wavy line), a wild-type *rosy* gene (▨), and a combination of DNA sequences from *E. coli* and the *Drosophila melanogaster white* locus⁷ (□). The part of pCPI that is inserted into the *Drosophila* genome by P-factor-mediated transposition includes both *per*⁺ and *rosy*⁺ DNA, and is represented by I–I. pCPI was mixed with p π 25.1, which mobilizes the P-factor associated with pCPI in *trans*⁸, at a 5:1 concentration ratio (350 μ g ml⁻¹ final concentration), and the mixture was injected into *Drosophila* embryos of the genotype *y per*⁰; *ry*⁴². Germ-line integration of pCPI DNA sequences results in a heritable change in eye colour (from *rosy* to wild-type). Because both *per*⁺ and *rosy*⁺ DNA sequences are integrated as a unit, flies with *ry*⁺ eye colour were subsequently tested for expression of behavioural rhythms. (For further details, see text.)

transformation of rhythmic behaviour accompanied transformation of eye colour.

Figure 2 shows eclosion profiles for three strains of flies. Two aspects of circadian behaviour were tested: first, the ability of a population of flies to entrain to a light-dark cycle (12 h of light followed by 12 h of dark); and second, the ability of populations of flies to free-run, or continue to behave rhythmically and synchronously, in the absence of the entraining light-dark cycle. In the experiments of Fig. 2, flies were kept in constant darkness from day 6 until the end of the experiment.

Figure 2a shows that flies carrying one dose of a wild-type (Oregon-R) *per* locus (*y per*⁰/+; *ry*⁴²/+ females) entrain to a light-dark cycle and continue to show circadian eclosion rhythms in constant darkness. The period of these rhythms is ~24 h during entrainment, and 25 h in constant darkness (see Fig. 2 legend). As expected^{1–3}, *per*⁰ flies (*y per*⁰/Y; *ry*⁴²/*ry*⁴² males, and *y per*⁰/*y per*⁰; *ry*⁴²/*ry*⁴² females) eclose arrhythmically in both sets of conditions (Fig. 2b). Flies in the population designated *per*⁰; P1.48C (Fig. 2c) differ from those in the *per*⁰ population only by the presence of the transforming DNA on one of their second chromosomes, at 48C (*y per*⁰/Y; P1.48C/+;

Fig. 2 Temporal profiles of eclosion (emergence of adults from pupal cases) for populations of: *a*, wild-type females ($y\ per^0/+; ry^{42}/+$); *b*, per^0 males and females ($y\ per^0; ry^{42}/ry^{42}$); and *c*, $per^0; P1.48C$ males and females ($y\ per^0; P1.48C/+; ry^{42}/ry^{42}$). Eclosion was monitored simultaneously in the three different populations by manually collecting (at 2-h intervals) the adults that had emerged in culture bottles maintained at 25 °C. Developing cultures were entrained to a cycle of 12 h light/12 h dark for 3 days before the start of collections. This light-dark (LD) cycle was continued for the first 5 days of collections, then cultures were maintained in constant darkness (DD) for the remainder of the experiment. The LD to DD transition is indicated by the arrow above record *a*. Collections during DD were made under a low-intensity safelight (a 15 W incandescent light with a Kodak GBX-2 filter) which does not affect *Drosophila* circadian rhythms^{1,2}. During entrainment to LD 12:12, 24-h period lengths were estimated for the rhythms of wild-type (*a*) and $per^0; P1.48C$ (*c*) populations by measuring the time elapsed between medians of successive peaks of eclosion. By the same procedure, wild-type and $per^0; P1.48C$ populations have free-running period lengths (in DD) of 25 ± 1.1 h and 26.5 ± 1.9 h, respectively. Similar estimates (24–25 h for wild-type and 27–28 h for $per^0; P1.48C$) were obtained from a spectral analysis of the data (Univariate Spectral Analysis BMDP P1T, University of California, Los Angeles; see ref. 11 for methods). Spectral analysis revealed no periodicity in the eclosion profile for per^0 flies.

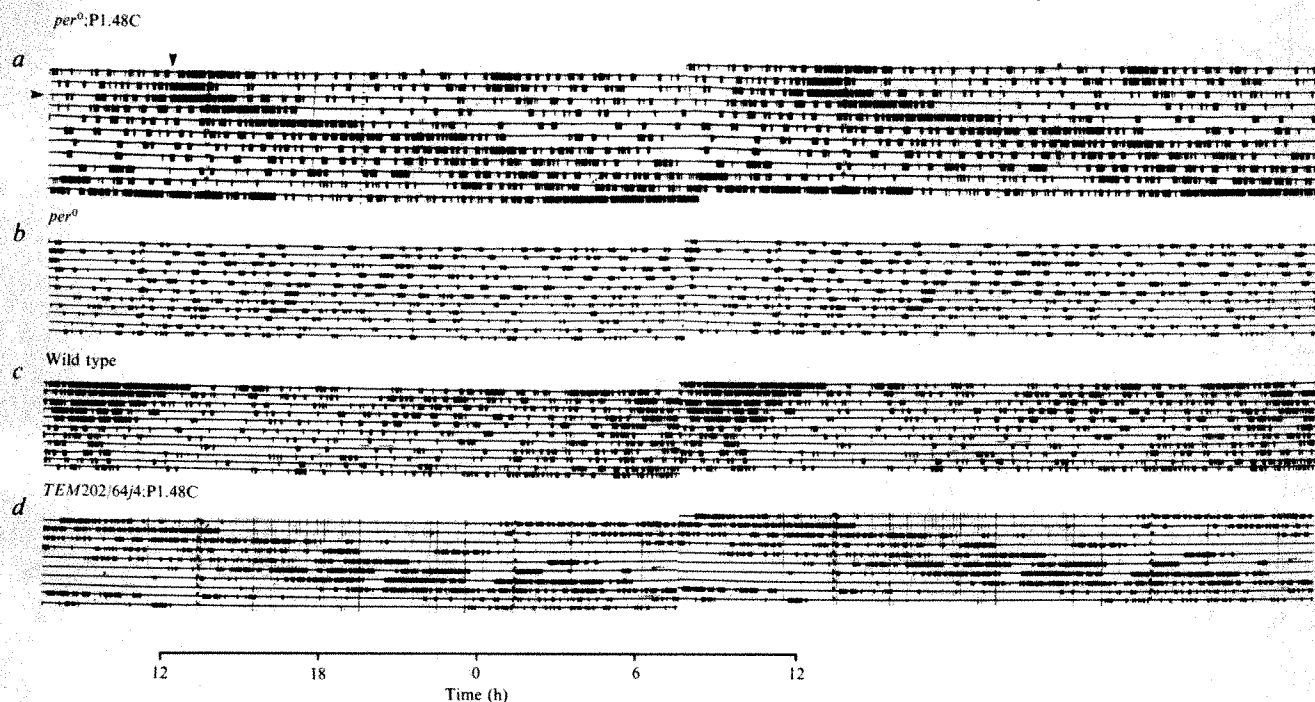
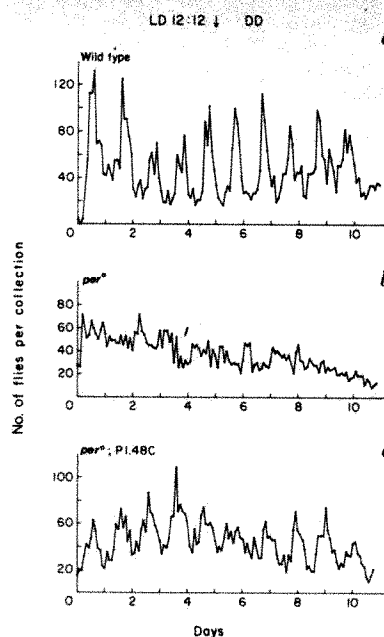


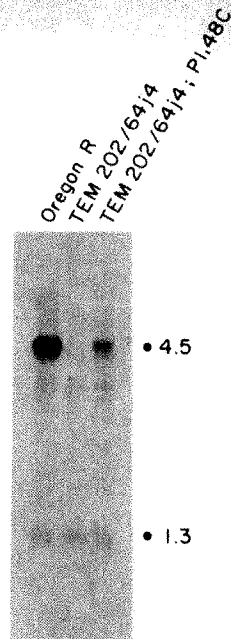
Fig. 3 Records of locomotor activity from individual flies: *a*, $y\ per^0/Y; P1.48C/+; ry^{42}/ry^{42}$ male; *b*, $y\ per^0/y\ per^0; ry^{42}/ry^{42}$ female; *c*, Oregon-R wild-type male; and *d*, $Df(1)TEM202/Df(1)64j4; P1.48C/+$ female. Data were collected by maintaining individuals, at 25 °C, in cylindrical plastic tubes (containing a small amount of medium) positioned between a light-emitting diode and a phototransistor of the monitoring device¹². The diode used emits light in a range (peak emission 900 nm) that does not affect circadian rhythms of locomotor activity¹². When a fly's movement disturbed the light beam, an all-or-none signal was generated which, when amplified, triggered a pen deflection on an Esterline Angus event recorder. Records were constructed by plotting successive days (24 h) of activity one below the other, then plotting twice the resulting chart of activity¹³. Thus, each record is shown twice, but the second record in every pair is displaced upwards 1 day on the right so that a continuous 48-h interval is shown on each horizontal line. In *a*, the first 3 days of the record show entrainment of the $per^0; P1.48C$ fly to a cycle of 12 h light/12 h dark (LD 12:12). Lights were switched on at 0 and off at 12 h. The final 'lights off' signal of the LD cycle is indicated by the intersection of the two arrows (12.00 on day 3). Thereafter, the fly remained in continuous darkness. For *b*, *c* and *d*, only activity in constant darkness is plotted. Period length was determined by measuring the time elapsed between offsets (cessations) of daily bouts of activity¹. All flies were entrained to LD 12:12, with 'lights on' and 'lights off' at the times indicated above, for at least 2 days before each activity recording began.

ry^{42}/ry^{42} males, and $y\ per^0/y\ per^0; P1.48C/+; ry^{42}/ry^{42}$ females). In sharp contrast to per^0 flies, $per^0; P1.48C$ flies eclose rhythmically during entrainment and continue to eclose rhythmically in constant darkness (Fig. 2c). The period of eclosion rhythms during entrainment is 24 h, whereas the period of free-running rhythms is ~27 h (see Fig. 2 legend).

Representative locomotor activity records for individual $per^0; P1.48C$, per^0 and Oregon-R flies are shown in Fig. 3; each

horizontal line of each record represents 48 h. Individual $per^0; P1.48C$ adults entrain to a light-dark cycle and continue to behave rhythmically in constant darkness (Fig. 3a). The first three days of the activity record in Fig. 3a ($y\ per^0/Y; P1.48C/+; ry^{42}/ry^{42}$ male) illustrate entrainment to a cycle of 12 h of light followed by 12 h of dark. Bursts of locomotor activity occur at dawn and dusk. The light-dark cycle ends on day 3 at 12.00. Bouts of activity in constant darkness continue to occur rhythmically.

Fig. 4 Transcriptional activity of P1.48C. Poly(A)⁺ RNAs from flies of the genotypes shown (5 µg per lane) were electrophoresed through 1% agarose/2.2 M formaldehyde gels and transferred to Biodyne A membrane (Pall Ultrafine Filtration). The 7.1-kb *Hind*III fragment from phage ZW106 (Fig. 1) was cloned into the Riboprobe vector pSP64 (Promega Biotec). The autoradiogram shows results from the hybridization of a single-stranded, ³²P-labelled, 1.5-kb RNA probe. The probe was initiated at the SP6 promoter of the vector and terminates at a *Bst*EII site within the 7.1-kb *Hind*III fragment; this *Bst*EII site is located 1 kb to the left of the rightmost *Bgl*III site shown in the ZW106 map of Fig. 1. This probe labels two RNAs after washing at 65 °C in 5 mM NaCl, 2 mM NaPO₄, pH 7, 0.1 mM EDTA, 0.1% SDS. The 1.3-kb RNA (indicated) is present in all samples, including flies deficient for 10 kb of DNA lying between the breakpoints of *Df(1)TEM202* and *Df(1)64j4*. As these flies lack the 7.1-kb *Hind*III fragment, the 1.3-kb RNA cannot be transcribed from this DNA⁶. The 4.5-kb RNA is absent from these flies, but is present in wild-type flies (Oregon-R), and is found in reduced amounts in flies of the genotype *Df(1)TEM202/Df(1)64j4*; P1.48C/+.



A densitometric analysis of shorter exposures of the autoradiograph indicates a 10-fold difference in the level of the 4.5-kb RNA between *Df(1)TEM202/Df(1)64j4*; P1.48C/+ and wild-type flies; a fivefold reduction from wild type would be expected in flies carrying two copies of P1.48C. Because the hybridization probe in these experiments is single-stranded, the direction of the transcription of the 4.5-kb RNA is from left to right along the 7.1-kb *Hind*III fragment shown in Fig. 1. Hybridizations using labelled RNA probes synthesized from the 7.1-kb *Hind*III fragment cloned in the opposite orientation fail to detect any homologous RNA species in the three genotypes shown.

cally with a period of ~27 h; each bout of activity occurs ~3 h later than that of the preceding day. Comparable results were obtained with 7 of 8 additional *per*⁰;P1.48C transformants, each monitored for a minimum of five free-running daily cycles (mean period = 28.1 ± 1.2 h). Thus, both eclosion rhythms and locomotor activity rhythms have approximately the same period.

Figure 3b shows the locomotor activity of a *per*⁰ fly (*y per*⁰/*y per*⁰; *ry*⁴²/*ry*⁴² female) in constant darkness. As with the eclosion behaviour of *per*⁰ populations, no rhythmic activity can be seen. Figure 3c shows a representative locomotor activity record of a free-running Oregon-R fly (male); the period of the rhythm in constant darkness is slightly less than 24 h. The period of the rhythms of such euploid, Oregon-R males is about an hour shorter than that of females carrying a single dose of *per*⁺ (Fig. 2); this result is expected because *per* is X-linked and dosage-compensated⁴, and period length is sensitive to *per* locus dosage⁴.

Df(1)TEM202/Df(1)64j4 females are arrhythmic and homozygously deficient for a 10-kb interval that includes the 7.1-kb *Hind*III fragment⁶. To determine whether the transforming DNA sequence in P1.48C can genetically complement this *per* locus deletion, a new group of flies (*Df(1)TEM202/Df(1)64j4*; P1.48C/+ females) was constructed. Figure 3d shows a representative locomotor activity record for a free-running *Df(1)TEM202/Df(1)64j4*;P1.48C fly; the fly is rhythmic, the period of the rhythm in constant darkness being 27 h. Of 25 flies tested, 24 produced comparable rhythms, with a mean period of 27.6 ± 2.4 h. No measurable difference could be found between the rhythms of *per*⁰;P1.48C and *Df(1)TEM202/Df(1)64j4*;P1.48C flies.

A 4.5-kb poly(A)⁺ RNA is transcribed from the 7.1-kb *Hind*III fragment in wild-type flies⁶. To determine whether the P1.48C

transforming DNA produces this RNA, two lines of *Df(1)TEM202/Df(1)64j4* aneuploid flies were constructed, one carrying the transposon insertion (that is, *Df(1)TEM202/Df(1)64j4*; P1.48C/+). Figure 4 shows that the 4.5-kb RNA is expressed in aneuploid flies only if P1.48C is present; it also demonstrates that the 4.5-kb RNA is much more abundant in wild-type than in *Df(1)TEM202/Df(1)64j4*; P1.48C flies (see Fig. 4 legend also).

Our analysis of *Drosophila* strain *per*⁰;P1.48C proves that a 7.1-kb *Hind*III fragment derived from a wild-type fly contains a functional *per* locus, and behavioural analysis of *Df(1)TEM202/Df(1)64j4*;P1.48C flies shows that the transforming DNA can complement *per* locus deletions. *Df(1)TEM202/Df(1)64j4*;P1.48C flies are still completely deficient for two transcription units neighbouring the 7.1-kb *Hind*III fragment; these map near the *T(1;4)JC43* chromosomal rearrangement breakpoint and code for a 1.1- and a 3.2-kb poly(A)⁺ RNA⁶. Because *Df(1)TEM202/Df(1)64j4*;P1.48C flies are rhythmic, it can be concluded that neither transcript has an essential role in the production of circadian behavioural rhythms.

The 7.1-kb *Hind*III fragment was chosen for these transformation experiments because it appeared to contain a single transcription unit that forms a 4.5-kb RNA⁶. As the 4.5-kb poly(A)⁺ RNA is in fact produced by the P1.48C transposon insertion, this RNA appears to be required for expression of behavioural rhythms.

As judged by an analysis of eclosion and locomotor activity rhythms, P1.48C restores the ability of *per*⁰ flies to entrain to light-dark cycles and to express rhythmic behaviour in constant darkness. The only measurable difference between the rhythms of wild-type and *per*⁰;P1.48C flies is the length of the free-running period. The finding that P1.48C transformants have long-period free-running rhythms is not particularly surprising given that period length is known to be sensitive to *per* locus dosage, with a 50% reduction in wild-type gene dosage increasing free-running rhythmic period length by 1 h. Because the level of expression of transforming DNA can be strongly influenced by chromosomal location in *Drosophila*^{9,10}, we suspect that P1.48C produces less than wild-type levels of a functional *per* gene product. Consistent with this interpretation, we find that *Df(1)TEM202/Df(1)64j4*;P1.48C/+ flies produce 10 times less than the wild-type level of 4.5-kb RNA. An analysis of the relationship of rhythmic period length and abundance of the 4.5-kb RNA among several strains of flies independently transformed with the 7.1-kb *Hind*III fragment is under way.

We thank Julie Pan for technical assistance, Laurel Eckhardt, Simon Kidd and Norton D. Zinder for criticism of the manuscript, Joseph Grossfeld for the loan of a micromanipulator for DNA injection experiments, and John Lis for instruction in microinjection techniques. This work was supported by grants from the Andre and Bella Meyer Foundation and the NIH to M.W.Y.

Note added in proof: A second *Drosophila* strain independently transformed with the 7.1-kb *Hind*III fragment, produces rhythms with close to wild-type periodicity. Of 15 such flies tested, 14 produced locomotor activity rhythms with a period 1 h shorter than flies transformed by P1.48C. A molecular analysis of *per* (ref. 6) has also been initiated by Reddy *et al.*¹⁴.

Received 5 October; accepted 22 October 1984.

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Influence of the method of drug administration on analgesic response

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The appropriate control group in studies of placebo-induced analgesia has not been established. A traditional control has been a 'no treatment' or natural-history group. In some studies, the natural-history group receives a hidden infusion of vehicle, a physiologically inactive substance such as saline solution, to eliminate differences in expectation of the outcome on the part of the experimenter^{1,2}. To evaluate whether 'hidden' as well as open infusion of vehicle can elicit a placebo response, we have now tested a different natural-history group, one which received an infusion of vehicle from a syringe pump controlled by a programmable timer. A comparison of these two control groups provides evidence that hidden infusion of vehicle can elicit a placebo response. Use of this new control group also permitted a clear distinction between a naloxone-antagonizable component of placebo analgesia and naloxone antagonism of endorphin-mediated analgesia induced by surgical stress. Our study underscores the power of the placebo and emphasizes that even the most subtle cues can elicit a placebo response.

Ninety-six patients underwent standardized surgery for the removal of impacted third molars. (The surgical procedure has been described in detail elsewhere^{3,4}.) The duration of the experiment, measured from the onset of local anaesthesia, was 5 h. Each patient was randomly assigned to receive, after surgery, a double-blind injection of one substance—naloxone, naloxone vehicle or morphine—via an indwelling intravenous line. Naloxone (10 mg) or naloxone vehicle were each administered to three groups of 12 patients. Each group received the randomly assigned substance by one of three methods: (1) by a person at the patient's bedside (open infusion, patient aware that a substance was being administered); (2) by a person in an adjacent room ('hidden' infusion^{1,2}); or (3) by the preprogrammed infusion pump (machine infusion). Morphine, included to assess the relative potency of placebo, was administered by machine infusion to two groups of 12 patients, one group receiving 8 mg and the other 12 mg of the drug. The average time of substance administration, 3 h after onset of anaesthesia, was the same for all groups. The effect of each intervention was determined as the change in pain 50 min after administration with respect to the pain intensity 10 min before administration. For the present study we have defined the natural-history group as those patients who received vehicle by machine infusion, placebo as the open administration of vehicle, and placebo analgesia as a decrease in pain, in comparison with the natural history group, produced by a placebo.

Mean pain intensity decreased similarly in the two groups receiving either open or hidden infusion of vehicle, but increased after machine infusion of vehicle (Fig. 1). Mean pain also increased after administration of naloxone, regardless of the route. Finally, pain decreased after machine infusion of both doses of morphine.

To demonstrate a naloxone-antagonizable component of placebo-induced analgesia, a 2×2 [(substance: naloxone, vehicle) $\times$ (route of administration: machine, open)] analysis of variance was performed. There was a significant interaction between substance and route of administration ($F(1, 44) = 8.07$, $P < 0.01$). Planned orthogonal comparisons of group means⁵ showed that pain was significantly decreased after open vehicle (placebo) ($F(1, 44) = 15.87$, $P < 0.001$) in comparison with the machine-infused vehicle (natural history) and naloxone groups

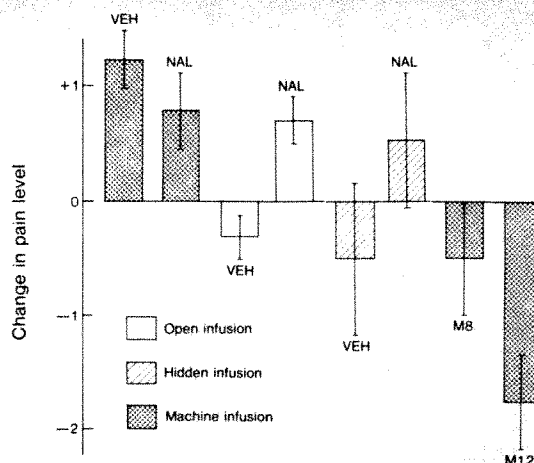


Fig. 1 Changes in pain intensity (mean $\pm$ s.e.) 50 min after the administration of morphine (M), vehicle (VEH) or naloxone (NAL), as compared with pretreatment baseline. Pain was measured with a 10-cm visual-analogue scale^{10,11} every 20 min after surgery. The position of the mark, in millimetres, provided the pain measure. The three methods of administration were open, 'hidden' and machine infusion. Pretreatment baseline pain intensities were: naloxone, open 4.9, hidden 5.8 and machine 6.1; vehicle, open 6.5, hidden 6.0 and machine 6.2; and morphine (machine only) 8 mg (M8) 6.2, and 12 mg (M12) 5.2. There were 12 patients in each group.

(Fig. 1). The significant decrease in pain in the open-vehicle group, in comparison with machine-infused vehicle, demonstrates that a placebo response was elicited. Pain intensity in the three groups that received naloxone and the group that received machine-infused vehicle did not differ significantly ($F(1, 44) = 0.85$, not significant; NS), and route of administration had no effect on the naloxone groups ($F(1, 44) = 0.010$, NS). In other words, pain was significantly increased after the administration of naloxone in comparison with open administration of vehicle, but not in comparison with machine infusion of vehicle.

Open infusion of naloxone produced an increase in pain whereas open infusion of vehicle produced a decrease, suggesting that there is a naloxone-antagonizable component of placebo-induced analgesia. That machine infusion of naloxone had no hyperalgesic effect relative to machine infusion of vehicle demonstrates that the higher pain intensity after open naloxone, in comparison with open vehicle, is due to naloxone antagonism of placebo-induced analgesia and is not an effect of naloxone on the natural history of post-operative pain. Our previous report that naloxone has a hyperalgesic effect³ may be explained also by the fact that we did not include an appropriate natural-history group.

Pain level in the hidden-vehicle group was also significantly less than in the group receiving vehicle by machine infusion ($F(1, 44) = 6.55$, $P < 0.02$). It did not differ significantly from the pain level in the openly-infused vehicle (placebo) group ($F(1, 44) = 0.03$, NS). These data suggest that hidden infusion of vehicle may be accompanied by unintentional cues that elicit a placebo response, whereas machine infusion of vehicle does not provide such cues. Surprisingly, in post-experiment interviews, patients were unable to identify cues signalling when hidden infusions had occurred or what substance had been infused. Thus, subtle cues, of which a patient may not be consciously aware, can significantly influence therapeutic outcome.

The magnitude of the analgesia produced by 8 mg machine-infused morphine was indistinguishable from that produced by placebo ($F(1, 88) = 0.024$, NS). Machine infusion of 12 mg morphine produced a decrease in pain, however, significantly greater than that produced by placebo ($F(1, 88) = 4.08$, $P < 0.05$)⁶. These observations together suggest that the potency of our placebo (open vehicle) is equivalent to approximately 8 mg

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morphine. This equivalent is larger than we reported in an experiment in which hidden infusion of morphine was used⁷. The difference between that experiment and the present one can presumably be explained by a summation of morphine and placebo effects introduced during hidden infusions.

In conclusion, the present study demonstrates that placebo-induced analgesia for post-operative pain is potent, and can be antagonized by naloxone, a relatively specific opiate antagonist⁸. This finding confirms our earlier study⁹, in which we did not have a natural-history group. Patients were categorized, based on their response to a placebo, as responders or non-responders. We inferred a contribution of endogenous opioids to placebo-induced analgesia from the observation that naloxone produced a greater increase in pain in placebo responders than in non-responders. In a study in which the natural-history group received a hidden infusion of naloxone, Gracely *et al.*² found that naloxone increased the pain in this control group, and concluded that naloxone had antagonized, not a placebo-induced analgesia, but a stress-induced, endorphin-mediated

analgesia occurring after surgery. Based on this conclusion, naloxone should also have significantly increased pain in their placebo group, but this was not observed. It is thus presently not possible to explain differences between our studies and that of Gracely and colleagues.

Importantly, this study also demonstrates that placebo-induced analgesia may be elicited by subtle cues of which a patient or experimental subject is unaware. Therefore, in studies where it is necessary to control for placebo effects, machine infusion of vehicle will allow the experimenters to preserve the same expectation of a therapeutic outcome for both experimental and control subjects.

We thank Professor Howard Fields for support and encouragement, Franklin Perry and Stephen Hinshaw for statistical consultation, Evangeline Leash for editorial assistance, Allan Basbaum for discussion of the manuscript, and Patricia Nishimoto and Marsha Stevens for technical assistance. This work was supported by NIH grants DE05369 and AM32634. J.L. is a Hartford Foundation Fellow.

Received 18 June; accepted 4 October 1984.

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Inhibition of aldosterone production in the adrenal glomerulosa by atrial natriuretic factor

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Several forms of the polypeptide atrial natriuretic factor (ANF) have been isolated recently from rat and human atria and identified¹⁻³; they are probably associated with the secretory granules of atrial tissue⁴. The potent ability of ANFs to increase urine sodium content is mediated by their direct action on the kidney^{5,6}. We report here the high intrinsic activity of a synthetic replicate of one form of this molecule, ANF(8-33) (ref. 7), to inhibit directly basal aldosterone secretion and its ability to antagonize the stimulatory effects of adrenocorticotropin (ACTH) and angiotensin II (AN-II) on the secretion of aldosterone by rat adrenoglomerulosa cells *in vitro*. Our results suggest that ANF is of clinical importance in the management of aldosterone-dependent hypertension by modifying the adrenocortical response to endogenous ACTH and AN-II.

Rat adrenoglomerulosa cells were obtained by enzymatic digest of adrenals collected from peripubertal male Sprague-Dawley rats (Holtzman, 125 g) using methods described previously⁸⁻¹⁰ (see Fig. 1). Synthetic ANF(8-33) has dramatic effects on the basal secretion of aldosterone by rat glomerulosa cells (Fig. 1). In concentrations as low as $\leq 10^{-9}$ M, there was a significant ($P < 0.01$) inhibition of aldosterone synthesis, even more accentuated at the higher concentrations tested ($P < 0.001$). Similar inhibitory effects on aldosterone formation were obtained when its secretion by the glomerulosa cells *in vitro* was stimulated with either ACTH (Fig. 2a) or AN-II (Fig. 2b). In each case, the simultaneous addition of equimolar amounts of ACTH or AN-II and ANF(8-33) resulted in a significant inhibition of aldosterone secretion ($P < 0.001$) when compared with that obtained by ACTH or AN-II alone. In these samples, corticosterone formation was inhibited by 10^{-7} M ANF(8-33), resulting in decreases from 24.7 ± 0.8 to 7.8 ± 3 ng ml⁻¹ ($P < 0.001$) in basal corticosterone formation, 37.2 ± 1.4 to $21.9 \pm$

0.9 ng ml⁻¹ ($P < 0.001$) on AN-II-stimulated corticosterone formation and 43.4 ± 0.7 to 36.1 ± 0.7 ng ml⁻¹ ($P < 0.001$) in ACTH-stimulated corticosterone formation, suggesting that ANF acts early in the steps of steroidogenesis, possibly as early as, or even before, cholesterol side-chain cleavage. The effect of ANF has also been demonstrated under various experimental designs, including increasing amounts of stimulus with ACTH and/or AN-II (results not shown). The specificity of ANF action on adrenal glomerulosa cells is demonstrated by its inability to modify basal or ACTH-stimulated corticosterone synthesis by rat fasciculata cells (inner zone after decapsulation) in suspension (Table 1). In similar studies using bovine fasciculata-reticularis cells¹¹ ANF(8-33) did not modify basal or ACTH-stimulated cortisol production. The amounts of cortisol formed by cells treated with 10^{-9} M ACTH(1-39) (40.8 ± 1.3 ng per well, $n = 12$) were not different from those formed by cells incubated with ACTH (10^{-9} M) and ANF(8-33) (10^{-9} M) (44.4 ± 1.1 ng per well, $n = 12$). Basal cortisol formation (0.12 ± 0.01 ng per well, $n = 12$) was also unaffected by ANF(8-33) (10^{-9} M) treatment (0.10 ± 0.01 ng per well, $n = 12$). In other experiments ANF(8-33) had no effect on pituitary ACTH secretion, demonstrating clearly a specific locus of action on the adrenal glomerulosa.

The observation that a synthetic replicate of ANF has the intrinsic activity to modify directly, at the glomerulosa cell level, the pattern of aldosterone secretion provides the first evidence for a humoral link between the heart and the adrenal cortex. While somatostatin^{12,13}, β -endorphin¹⁴ and met-enkephalin¹⁵ have been associated with similar biological activities, their physiological significance is uncertain. The potency of ANF

Table 1 Specificity of the effect of ANF(8-33) on adrenal steroidogenesis

Treatment	Corticosterone (ng ml ⁻¹)
Control	81 $\pm$ 6
ANF(8-33)	91 $\pm$ 8
ACTH(1-39)	782 $\pm$ 27
ANF(8-33) + ACTH(1-39)	752 $\pm$ 16

Enzymatic digests of cells were prepared from the inner zone of decapsulated adrenals obtained from normal male (125 g) rats, as described in Fig. 1 legend. All treatments were added at a concentration of 10^{-9} M.

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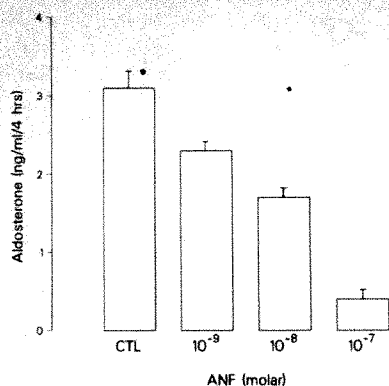


Fig. 1 Effect of ANF(8-33) on basal aldosterone secretion. Rat glomerulosa cells were prepared by enzymatic digestion of 20 rat adrenals after enucleation. The cells remaining on the capsule were digested for 30 min with a mixture of collagenase and DNase (4 mg ml^{-1} , $4 \mu\text{g ml}^{-1}$) for 30 min. Dispersed cells were filtered through gauze and centrifuged at 800 r.p.m. for 15 min. The pellet was resuspended in M199 buffer containing 0.1% bovine serum albumin (BSA) and the cells centrifuged at 800 r.p.m. for 15 min. The cell pellet was again resuspended in M199-0.1% BSA buffer and distributed in 900- μl aliquots to 12 $\times$ 75 plastic tubes. The samples were preincubated for 90 min in a 37°C waterbath under an atmosphere of 5% CO_2 /95% O_2 . Aliquots of the test samples were added in a 100 μl volume and incubated for 4 h. Aldosterone and corticosterone were measured by radioimmunoassay using antisera purchased from Endocrine Sciences, Oxnard, California, and ^3H -labelled steroid from NEN. Results are the mean $\pm$ s.e.m. of seven replicates. Statistical analysis was performed by analysis of variance and all points are significant ($P < 0.01$) from control. (ANF(8-33) was the gift of Drs R. Hirschmann and D. F. Veber of Merck, Sharp and Dohme Research Laboratories).

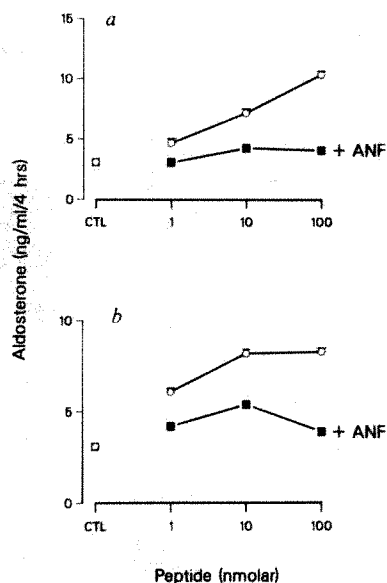


Fig. 2 Effect of ANF(8-33) on stimulated aldosterone secretion. **a**, Rat glomerulosa cells were prepared as described in Fig. 1 and incubated with synthetic human ACTH either alone (open circles) or in combination with equimolar amounts of ANF(8-33) (closed squares). Aldosterone secretion was measured as above by radioimmunoassay. **b**, Cells were incubated with synthetic angiotensin-II (AN-II) either alone (open circles) or in the presence of equimolar amounts of ANF(8-33) (closed squares). Control cells received neither peptide, thereby indicating the ability of ANF to decrease aldosterone production to basal levels. Results are the mean $\pm$ s.e.m. of 7 replicates and all points are significant when compared with their respective control ($P < 0.001$). Synthetic hACTH(1-39) and angiotensin II were synthesized by Dr Nicholas Ling by solid-phase methodology.

(8-33) as a natriuretic hormone, and now in inhibiting basal and stimulated aldosterone formation, suggests that its biological activities are an integral part of the homeostatic mechanisms regulating sodium retention. Furthermore, unlike somatostatin, its inhibitory effect is not restricted to angiotensin-stimulated aldosterone secretion, but affects the formation of both basal and stimulated mineralocorticoids. Moreover, at no point was ANF(8-33) observed to stimulate aldosterone. The observations reported here provide the groundwork for defining the mechanisms by which atrial-derived peptides affect sodium retention and suggest that this peptide may be responsible for the attenuated effects of AN-II on the adrenal cortex during sodium loading^{16,17}. The understanding of some clinical forms of idiopathic hypo- and hypertension^{18,19} may therefore result from defining the interactions between ANF, the adrenal cortex and the basic mechanisms regulating ANF secretion.

After submission of this manuscript, Chartier *et al.*²⁰ and DeLean *et al.*²¹ reported findings similar to those reported here.

We thank Drs R. Guillemin and P. Böhlen for their critical review and comments on this manuscript and the secretarial staff of the Laboratories for Neuroendocrinology for help in preparation of the manuscript. This research was supported by grants from the NIH (HD-09690 and AM-18811) and the Robert J. Kleberg Jr. and Helen C. Kleberg Foundation.

Received 18 May; received in revised form 16 July; accepted 15 November 1984.

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Molecular cloning of lymphadenopathy-associated virus

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Lymphadenopathy-associated virus (LAV) is a human retrovirus first isolated¹ from a homosexual patient with lymphadenopathy syndrome, frequently a prodrome or a benign form of acquired immune deficiency syndrome (AIDS)². Other LAV isolates have subsequently been recovered from patients with AIDS or pre-AIDS³⁻⁵ and all available data are consistent with the virus being the causative agent of AIDS. The virus is propagated on activated T lymphocytes and has a tropism for the T-cell subset OKT4 (ref.

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6), in which it induces a cytopathic effect. The major core protein of LAV is antigenically unrelated to other known retroviral antigens^{1,2,7}. LAV-like viruses have more recently been independently isolated from patients with AIDS and pre-AIDS. These viruses, called human T-cell leukaemia/lymphoma virus type III (HTLV-III)⁸⁻¹¹ and AIDS-associated retrovirus (ARV)¹², seem to have many characteristics in common with LAV and probably represent independent isolates of the LAV prototype. We have sought to characterize LAV by the molecular cloning of its genome. A cloned LAV complementary DNA was used to screen a library of recombinant phages constructed from the genomic DNA of LAV-infected T lymphocytes. Two families of clones were characterized which differ in a restriction site. The viral genome is longer than any other human retroviral genome (9.1–9.2 kilobases).

The cDNA first-strand of LAV was synthesized in an endogenous, detergent-activated reaction. LAV virions were purified from the supernatant of FR8 cells, a B-lymphoblastoid LAV-producing line¹³, and the reaction was primed with oligo(dT). Three cDNA clones, pLAV13, 75 and 82, carrying inserts of 2.5, 0.6 and 0.8 kilobases (kb), respectively, were characterized further (Fig. 1). All three inserts have a common restriction pattern at one end, indicative of a common priming site. The 50-base pair (bp) common *Hind*III–*Pst*I fragment was sequenced and shown to contain an oligo(dA) stretch preceding

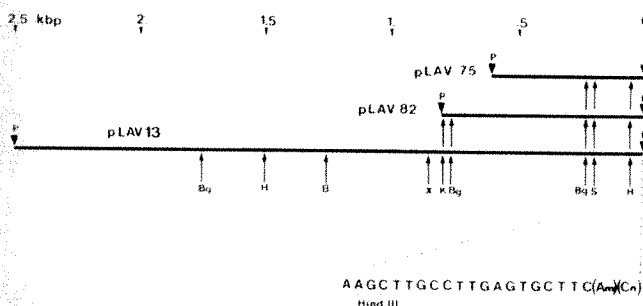


Fig. 1 Restriction maps of cDNA clones derived from LAV genomic RNA. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; K, *Kpn*I; S, *Sac*I; X, *Xho*I.

Methods: LAV cDNA was synthesized in an endogenous detergent-activated reaction. For each reaction, LAV virions were purified on a 20–60% sucrose gradient as described previously¹, from 200 ml of supernatant of the LAV-producing FR8 line¹³. Virus-containing fractions were pooled, diluted with NTE buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.8, 1 mM EDTA) and centrifuged (Beckman type SW56 rotor, 50,000 r.p.m., 60 min). The viral pellet was resuspended in 250 μ l of NTE. Reaction volume was adjusted to 1 ml and final concentrations were: 50 mM Tris-HCl pH 7.8, 25 mM NaCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.02% Triton X-100, 0.1 mM of each of dATP, dGTP, TTP, 4 μ M dCTP including 200 μ Ci of [α -³²P]dCTP (400 Ci mmol⁻¹, Amersham) and 50 μ g ml⁻¹ oligo(dT) primer. Incubation was at 37 °C. After 15 min, dCTP was added to 25 μ M. At 45 min, the reaction was stopped with EDTA and SDS (final concentrations 20 mM and 0.5%, respectively). After 1 h of proteinase K digestion (100 μ g ml⁻¹, 37 °C), the reaction mixture was extracted with phenol/chloroform and cDNA-RNA hybrids were ethanol-precipitated. Second-strand synthesis with nuclease-free DNA polymerase I (Boehringer) and RNase H (BRL) and dC-tailing with terminal transferase (Boehringer) were performed according to Gubler and Hoffman²⁶. Tailed double-stranded cDNA was annealed to dG-tailed *Pst*I-linearized pBR327 vector. *Escherichia coli* C600 recBC was transformed by the $CaCl_2$ method; 500 recombinant clones were screened *in situ*²⁷ with a ³²P-labelled LAV cDNA in which the first strand had been synthesized as described above, except that an alkaline hydrolysis step was included. Approximately 10% of recombinants proved positive, the majority of which formed a family of cross-hybridizing clones. Three recombinants, pLAV13, pLAV75 and pLAV82, carrying inserts of 2.5, 0.6 and 0.8 kb, respectively, were analysed further. There are no sites for *Eco*RI, *Nru*I, *Pvu*I, *Sal*I, *Sma*I, *Stu*I or *Xba*I in the pLAV13 insert. The *Hind*III–*Pst*I fragment was subcloned into M13mp8 and sequenced according to Sanger *et al.*²⁸ using a 15-mer primer (Biolabs) and [α -³²P]dCTP (Amersham).

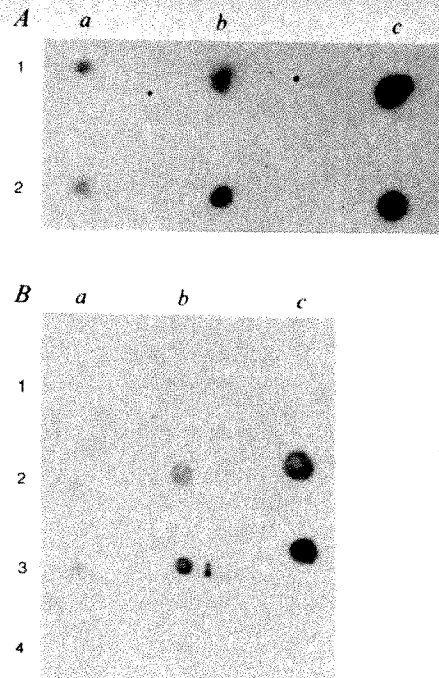


Fig. 2 Rapid dot-blot technique for LAV detection in cell culture supernatant. Spots represent: A, a, 1 μ l; b, 2 μ l; c, 4 μ l of concentrated (250 $\times$) cell culture supernatant from (1) LAV-producing CEM cells (reverse transcriptase activity (RT), determined as described previously¹, was 140,000 c.p.m. ml⁻¹); (2) LAV-producing Epstein-Barr-transformed B-cell line FR8 (RT 175,000 c.p.m. ml⁻¹). B, a, 1 μ l; b, 2 μ l; c, 5 μ l of 100 $\times$ concentrated supernatant from (1) uninfected normal T lymphocytes (no RT activity); (2) LAV-producing normal T lymphocytes (RT 170,000 c.p.m.); (3) LAV-producing CEM line (RT 150,000 c.p.m.); and (4) culture of bone marrow lymphocytes from a haemophiliac patient⁴ with AIDS (RT 7,000 c.p.m.).

Methods: Cell culture supernatants were pelleted through 0.5 ml 20% sucrose cushions in NTE buffer (Beckman type SW56 rotor, 50,000 r.p.m., 1 h, 4 °C). The pellet was resuspended in NTE buffer as indicated. Concentrated virus was spotted onto dried nylon filters (Zetabind) presoaked in 20 $\times$ SSC (3 M NaCl, 0.3 M sodium citrate). After baking (at least 30 min at 80 °C), filters were hybridized with ³²P nick-translated pLAV13 insert (Fig. 1) (specific activity >10⁸ c.p.m. per μ g) for 12–16 h in stringent conditions (50% formamide, 5 $\times$ SSC, 42 °C), washed (0.1 $\times$ SSC, 0.1% SDS, 65 °C, 2 $\times$ 30 min), and exposed for 20 h (Kodak XAR5 film with an intensifying screen) at –70 °C.

the cloning dC tail. The clones are thus copies of the 3' end of a poly(A) RNA.

The specificity of pLAV13 was determined in a series of filter hybridization experiments using nick-translated pLAV13 insert as a probe. First, using an adapted spot-blot technique, we could detect LAV virion RNA from normal T cells, FR8 and other B-cell lines and CEM cells (L.M. and R. Weiss, unpublished results; Fig. 2). LAV was also detected in a bone marrow cell culture (Fig. 2B, line 4) from a haemophiliac with AIDS⁴, in spite of the low titre of virus in the supernatant. Uninfected cultures proved negative (Fig. 2B, line 1). Second, the probe detected DNA in the Southern blots of LAV-infected T lymphocytes and CEM cells (Fig. 3). No hybridization was detected in DNA from uninfected lymphocytes or from normal liver (data not shown) in the same hybridization conditions. A characteristic 1.45-kb *Hind*III fragment which co-migrated with an internal viral fragment in *Hind*III-cleaved pLAV13 (Fig. 1) was detected in the Southern blots. Bands at 2.3 and 6.7 kb were also detected. Together, these data show that pLAV13 DNA is exogenous to the human genome and detects both RNA and integrated DNA forms derived from LAV-infected cells. Thus, pLAV13 is LAV specific. Being oligo(dT)-primed, pLAV13 must contain the R and U3 regions of the long terminal repeat (LTR) as well as

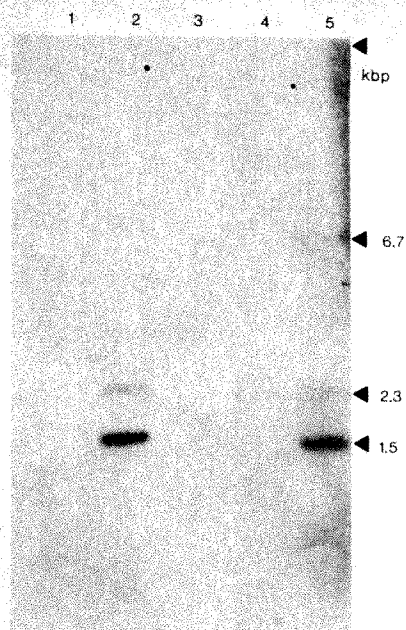


Fig. 3 Southern blot of *Hind*III-restricted genomic DNA from LAV-infected and uninfected cells hybridized with pLAV13. *Hind*III-restricted high relative molecular mass DNA from: lane 1, uninfected CEM cells; lane 2, LAV-infected CEM cells; lane 3, uninfected T cells after 5 days' culture; lane 4, LAV-infected T cells 2 days after infection; lane 5, LAV-infected T cells 5 days after infection.

Methods: Peripheral blood T lymphocytes of a healthy donor were stimulated for 3 days with phytohaemagglutinin, after which they were infected with LAV (isolate BRU-LAV1) at 10^4 c.p.m. reverse transcriptase activity per 10^6 cells as described previously¹, except for part of the culture kept uninfected for controls. Two and five days after infection, genomic DNA was extracted. *Hind*III-digested DNA (10 μ g) was electrophoresed through a 0.8% agarose gel and Southern blotted. The filter was hybridized in 10 ml of 50% formamide, 5 $\times$ SSC, 1 $\times$ Denhardt's, 10% dextran sulphate with 100 μ g ml⁻¹ denatured sonicated salmon sperm DNA and 2×10^7 c.p.m. of nick-translated pLAV13 insert (4×10^8 c.p.m. per μ g) for 10 h at 42 °C. The filter was washed at 68 °C in 0.1 $\times$ SSC, 0.1% SDS for 2 $\times$ 30 min and exposed to Kodak XAR5 film at -70 °C for 16 h using an intensifying screen.

the 3' end of the coding region, assuming a conventional retroviral genome structure.

Having found a *Hind*III site about 20 bp 5' of the poly(A) stretch and thus within the R region of the LTR, we cloned the LAV genome by making a partial *Hind*III digest of genomic DNA from LAV-infected T cells of a healthy donor. A 9 ± 1.5 -kb DNA-containing fraction was precipitated and ligated into the *Hind*III arms of phage vector λ L47.1 (ref. 14). When nick-translated pLAV13 insert was used as a probe to screen $\sim 2 \times 10^6$ phage plaques *in situ*, five independent clones were obtained. A restriction map of clone λ J19 and of a *Hind*III variant, λ J81, are shown in Fig. 4. Recombinants λ J27, λ J31 and λ J57 have the same *Hind*III map as λ J19, while λ J81 is so far unique. As the two clones were derived from the first isolate¹ of LAV reported (isolate BRU, or LAV1), we refer to the two viral genomes as LAV1a (λ J19) and LAV1b (λ J81). λ J19 shows four *Hind*III bands of 6.7, 1.45, 0.6 and 0.52 kb, the first two of which correspond to bands in the genomic blot of *Hind*III-restricted DNA (Fig. 3, lane 5). The smallest bands (0.6 and 0.52 kb) were not seen in the genomic blot, but the fact that they appear in all the independently derived clones analysed indicates that they represent internal and not junction fragments, assuming random integration of LAV proviral DNA. However, the 0.52-kb band hybridizes with pLAV13 DNA (Fig. 4) through the small *Hind*III-*Pst*II fragment of pLAV13. Thus, the 0.5-kb *Hind*III fragment of λ J19 contains the R/U5 junction within the LTR. The finding of two small *Hind*III fragments in the 5' region reinforces

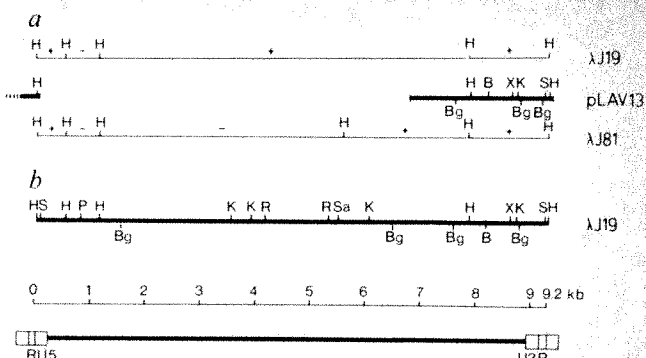


Fig. 4 Restriction maps of LAV proviral DNA in clones λ J19 (LAV1a) and λ J81 (LAV1b). *a*, *Hind*III restriction maps of LAV proviral DNA in clones λ J19 and λ J81. Those *Hind*III fragments detected by pLAV13 are marked by +, those not, by -. The restriction map of the pLAV13 cDNA clone is also shown. *b*, Restriction map of λ J19. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; S, *Sac*I; Sa, *Sal*I; X, *Xho*I. Beneath the scale is a schema for the general structure of retroviruses showing the LTR elements U3, R and U5. Only the R/U5 boundary has been defined (Fig. 1) and other boundaries are drawn only figuratively.

Methods: DNA from LAV-infected T cells was partially digested with *Hind*III and fractionated on a 5–40% sucrose gradient in 10 mM Tris-HCl pH 8, 10 mM EDTA, 1 M NaCl (Beckman type SW41 rotor, 16 h, 40,000 r.p.m.). A single fraction (9 ± 1.5 kb) was precipitated with 20 μ g ml⁻¹ dextran T40 as carrier and taken up in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). λ L47.1 (ref. 14) *Hind*III arms were prepared by first ligating the *cos* sites followed by *Hind*III digestion and fractionation through a 5–40% sucrose gradient as above. Fractions containing only the λ *Hind*III arms were pooled, precipitated and taken up in TE buffer. Ligation of arms to DNA was made at ~ 200 μ g ml⁻¹ DNA using a 3:1 molar excess of arms and 300 U of T4 DNA ligase (Biolabs). *In vitro* packaging lysates were made according to ref. 29. After *in vitro* packaging, the phage lysate was plated out on NM538 or a C600 recBC strain. Approximately 2×10^6 plaques were screened by *in situ* hybridization³⁰ using nitrocellulose filters. Hybridization was performed at 68 °C in 1 $\times$ Denhardt's solution, 0.5% SDS, 2 $\times$ SSC, 2 mM EDTA. Probe: ³²P nick-translated insert of pLAV13 at $> 10^8$ c.p.m. per μ g. Filters were washed for 2 $\times$ 30 min in 0.1 $\times$ SSC 10.1% SDS at 68 °C, and exposed to Kodak XAR-5 film for 24–40 h with intensifying screens at -70 °C. Seven positive clones were identified and plaque-purified on a C600 recBC strain. Liquid cultures were grown and the recombinant phages banded in CsCl. Phage DNA was extracted and digested in the appropriate conditions. The restriction maps were orientated by hybridizing blots to pLAV13 DNA, which maps the 3' coding sequences of the viral genome as well as the U3-R region of the LTR. All cloning and amplification of LAV genomic clones was carried out in a P3 laboratory.

the usefulness of cloning LAV by partial restriction of genomic DNA.

λ J81 seems to be a restriction site polymorph of λ J19, showing five *Hind*III bands of 4.3, 2.3, 1.45, 0.6 and 0.52 kb (Fig. 4). The 2.3-kb band is readily detected in the genomic blot by a pLAV13 probe, although the 4.3-kb fragment is not. The finding that nick-translated λ J19 DNA hybridizes to all five *Hind*III bands of λ J81 in stringent hybridization and washing conditions indicates that λ J81 is a *Hind*III variant and not a recombinant virus. Also, other mapped restriction sites in λ J81 are identical to those of λ J19 (not shown). Thus, the *Hind*III restriction pattern in the Southern blot can be explained by variation within the single isolate of LAV used to infect the T cells.

HTLV-I¹⁵ and HTLV-II¹⁶ constitute a pair of C-type transforming retroviruses with a tropism for the T-cell subset, OKT4. Both genomes (comprising one LTR) are ~ 8.3 kb long^{17,18}, have an X region and show extensive sequence homology. They hybridize between themselves in reasonably stringent conditions (40% formamide, 5 $\times$ SSC) and the X regions hybridize even at 60% formamide¹⁹. Thus, a conserved X region is a hallmark of

this class of virus. We have compared cloned LAV DNA and cloned HTLV-II DNA (pMO)²⁰ by blot-hybridization and find no cross-hybridization in low stringency conditions of hybridization and washing ($T_m = 55^\circ\text{C}$), even after 2 days' exposure at -70°C using intensifying screens (data not shown).

The human T-lymphotropic retroviruses HTLV-III⁸ and ARV¹², recently isolated from patients with AIDS or pre-AIDS, have similar morphological, biochemical and immunological properties to LAV, which suggests that they probably represent different isolates of the LAV prototype. DNA hybridization between HTLV-III and HTLV-I and -II has been reported, most noticeably at the *gag-pol* junction and less so in the characteristic X region of HTLV-I and -II²¹. As mentioned above, we could detect no such hybridization and conclude that the reported homology must have been due to either (1) the use of an uncloned cDNA as hybridization probe, (2) the fact that the isolates in question differ substantially from those we have cloned, or (3) the possibility that HTLV-III and a HTLV-I/II-like virus were co-infecting the cells. The last possibility may also apply to the preliminary report of cross-hybridization between a LAV-like virus and a cloned HTLV-II DNA probe⁷. Thus, we find no molecular evidence of a relationship between LAV and HTLV. Furthermore, the LAV genome is ~9 kb long, compared with 8.3 kb for the HTLV viruses^{17,18}. Despite their comparable genome sizes, LAV does not cross-hybridize with Visna virus²² (~9 kb) (data not shown) or with several human endogenous viral genomes (ref. 23 and M. Martin, personal communication) in non-stringent conditions ($T_m = 55^\circ\text{C}$). These data and morphological and immunological dissimilarities^{1,2} between LAV and the HTLV-I/-II pair all point to LAV being a novel class of human retrovirus.

In conclusion, we have molecularly cloned the complete genome of LAV from freshly infected activated T cells of a healthy donor. It has been shown that the tropism of certain retroviruses resides in the LTR^{24,25} and that sequence differences and insertions/deletions are present in the LTRs of leukaemogenic and non-leukaemogenic retroviruses. It is thus possible that LAV and LAV-like viruses passaged through B- and T-transformed cell lines^{8,12,13} might have undergone some attenuation. Although the cDNA clones were made from a LAV-producing B-cell line, the genomic clones were isolated from LAV-infected normal T cells. Thus, the clones represent LAV genomes that have not been selected or adapted to a particular cell line. However, the LAV genome is shown to be polymorphic even within a single isolate and independent isolates will probably differ widely.

The availability of cloned LAV DNA should facilitate the understanding of the molecular mechanism of viral replication, and the tropism of the virus. The DNA sequence of LAV opens up the possibility of expressing the viral *gag* and *env* gene products and of studying the molecular basis of LAV antigenicity.

We thank Drs D. Dormont and J. Weissenbach for their interest in this work, Denise Guetard, Sophie Chamaret and Jacqueline Gruet for cells, Dr R. C. Gallo for the HTLV-II probe (pMO), Dr M. Brahic for a cloned Visna probe (λ 109) and Ana Cova for typing the manuscript. This work was supported by grants from the CNRS, the Association pour la Recherche contre le Cancer, the Fondation pour la Recherche Médicale and Institut Pasteur.

Received 20 September; accepted 12 November 1984.

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Molecular cloning of AIDS-associated retrovirus

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Retroviruses cause a wide variety of diseases in avian and mammalian species. Human acquired immune deficiency syndrome (AIDS) leads to collapse of the immune system and death by a wide variety of opportunistic infections; unusual forms of cancer are associated with this syndrome. Retroviruses have been recovered from tissues of AIDS patients and from patients with related conditions. These similar newly-isolated viruses are lymphadenopathy-associated virus (LAV)¹, human T-cell lymphotropic virus (HTLV-III)^{2,3} and AIDS-associated retrovirus (ARV-2)⁴. We have identified a RNA genome of ~9 kilobases (kb) in virions purified from the culture medium of a human T-cell tumour line infected with ARV-2. A cDNA probe made from viral RNA detected circular DNA molecules and proviral forms in infected cells. We prepared a library of infected cell DNA. Recombinant phage included those with a 9.5-kb proviral DNA and viral DNA permuted with respect to the single *EcoRI* site. Comparison of three ARV isolates from different AIDS patients revealed polymorphism of restriction endonuclease sites.

HUT-78 cells, originating from a human T-cell lymphoid tumour⁵, were used to propagate the ARV-2 strain of virus⁴. To characterize the viral genome, RNA was extracted from purified virions and electrophoresed on agarose gels containing methyl mercury hydroxide⁶. A distinct ~9-kb RNA species was observed (Fig. 1) with smaller heterogeneous RNA and some ribosomal RNA species. The 9-kb RNA species was used as a template with random primers in a reverse transcriptase reaction to produce a virus-specific cDNA probe⁷. RNA of virus obtained from cells infected with ARV-2 or with two additional isolates, ARV-3 and ARV-4, showed distinct bands at 9 kb that hybridized with the cDNA probe (Fig. 1).

With this cDNA probe, we examined the structure of viral DNA in infected cells by digestion with restriction enzymes, electrophoresis in agarose gels and Southern blotting. No specific bands were detected in several digests of DNA from uninfected cells (Fig. 2a, lanes C, E), whereas bands were seen in infected cells (Fig. 2a, lane A). Undigested DNA from infected cells contained a species at 5.5 kb, a faint species at 6 kb

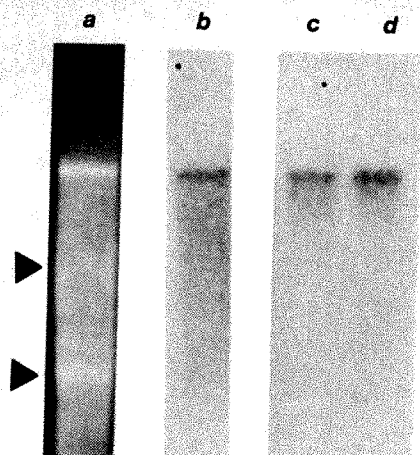


Fig. 1 Analysis of RNA from ARV. To produce an acute infection, the HUT-78 cells were inoculated with virus and fresh medium was added to maintain growing cultures at intervals of 2–3 days. Virus production was monitored by measuring reverse transcriptase activity with exogenous template in viral particles released into the culture medium⁴. After inoculation with tissue culture virus, progeny virus was detected at 3–4 days after infection and production peaked at 6–8 days. To purify virus particles, the culture medium was spun at low speed (1,000 r.p.m., 5 min, 4°C; all subsequent operations were at 4°C) to remove cells and further clarified by centrifugation at 7,000 r.p.m. for 10 min. This supernatant was centrifuged at 26,000 r.p.m. for 1 h in the SW28 rotor to pellet virus. The pellet from 100 ml culture medium was resuspended in 2 ml 10 mM Tris-HCl, pH 7.5. The resuspended virus pellet was treated with 10 $\mu\text{g ml}^{-1}$ DNase (Boehringer-Mannheim) for 1 min on ice and layered onto a 10–50% sucrose gradient (buffer containing 100 mM NaCl, 0.1 mM EDTA and 20 mM Tris-HCl, pH 7.6) and centrifuged in the SW41 rotor for 2 h at 34,000 r.p.m. Fractions were collected, extracted with phenol, and nucleic acid from the aqueous phase was adjusted to 300 mM NaCl and precipitated with cold ethanol. A sample of each fraction was electrophoresed on 1% agarose gels (containing 5 mM methyl mercury hydroxide)⁶ and stained with ethidium bromide to locate the fraction enriched for virion RNA (lane a). Arrows show the positions of ribosomal RNA markers at 4.9 and 1.9 kb. The remaining RNA from this gradient fraction was electrophoresed in low-melting 1% agarose gel (containing 5 mM methyl mercury hydroxide). The region in the gel containing the ~9 kb RNA species was cut out, melted at 70°C, and the RNA extracted with phenol and concentrated by precipitation with cold ethanol. We obtained 0.5–1 μg of purified 9 kb RNA from 100 ml of culture medium. To synthesize a radioactive cDNA probe, this RNA was used as template for reverse transcriptase with random DNA primers (calf thymus⁷) and with the addition of 20 $\mu\text{g ml}^{-1}$ actinomycin D to the reaction. RNA from ARV-2, ARV-3 and ARV-4, electrophoresed on 1% agarose gels (containing methyl mercury hydroxide), were transferred to nitrocellulose filters⁹ and hybridized with the cDNA probe (lanes b, c and d, respectively). These hybridizations were performed in 50% formamide, 3 $\times$ SSC at 42°C and washes were at 50°C in 0.2 $\times$ SSC.

and a broad band at the exclusion limit of the gel (>15 kb). We suggest that the DNA species at 5.5 and 6 kb represent unintegrated viral DNA in a circular configuration containing, respectively, one and two long terminal repeats (LTRs); the upper broad band (>15 kb) represents proviruses integrated into host cell DNA (Fig. 2a, lane A).

EcoRI digestion gave a 9.5-kb fragment, and *SacI* gave 5.7- and 3.8-kb fragments (Fig. 2a, lane D). Digestion of infected cell DNA with a mixture of *EcoRI* and *SacI* yielded DNA fragments at 5.4 and 3.8 kb (Fig. 2a, lane F). Digestions with *HindIII* (Fig. 4, *PvuII* or *BglII*) produced five or more DNA fragments (data not shown). The sum of the molecular weights of the viral DNA fragments in each digest was 9.2–9.5 kb. A restriction enzyme map of a proviral form in HUT-78 cells infected with ARV-2 was produced (Fig. 2).

A complex pattern was produced in *KpnI* digestions. A major species of 5.2 kb and a minor species of 4.2 kb were observed

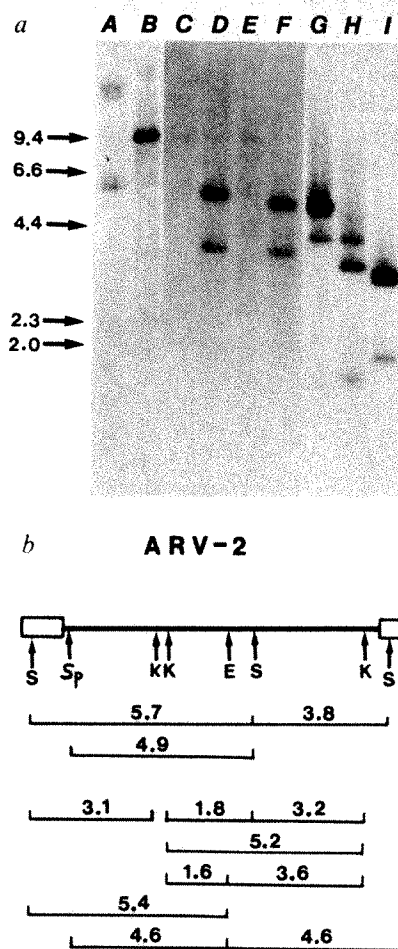


Fig. 2 Detection and organization of ARV DNA in infected cells. Whole-cell DNA was prepared by a phenol extraction method¹⁰ from uninfected HUT-78 cells and from cells 10 days after infection with ARV-2. These DNA preparations were analysed by restriction enzyme digestions, electrophoresis on 1% agarose gels coupled with transfer to nitrocellulose¹⁰ and annealing with radioactive viral cDNA probe⁷. Annealing was done at 42°C in 50% formamide and 3 $\times$ SSC, and washes were performed at 50°C in 0.2 $\times$ SSC. **a**, Lane A, undigested infected cell DNA; B, infected cell DNA, *EcoRI*; C, HUT-78 DNA, *SacI*; D, infected cell DNA, *SacI*; E, HUT-78 DNA, *EcoRI* and *SacI*; F, infected cell DNA, *EcoRI* and *SacI*; G, H, I, infected cell DNA digested with, respectively, *KpnI*, *KpnI* and *EcoRI*, and *KpnI* and *SacI*. Markers (in kb) are *HindIII* fragments from bacteriophage λ DNA. **b**, The positions of restriction enzyme sites and DNA fragment sizes. Open boxes, inferred LTR structures; S, *SacI*; E, *EcoRI*; K, *KpnI*; Sp, *SphI* site. The orientation of the map is based on DNA sequence information (our unpublished results).

(Fig. 2a, lane G). Digestion with a mixture of *KpnI* and *EcoRI* cleaved the 5.2-kb fragment to a 3.5 and a 1.7 kb species; the faint 4.2-kb species remained (Fig. 2a, lane H). *SacI* cleaved both of the *KpnI* fragments (Fig. 2a, lane I). These digestion patterns can be explained if the *KpnI*-generated 5.2-kb fragment is an internal proviral DNA fragment and the minor species is a circle junction from circular viral DNA possessing one LTR. Autoradiographs of the *KpnI* digest showed a very faint band at 5.1 kb not affected in the double digestion with *KpnI* and *EcoRI*; this 5.1-kb band disappeared after double digestion with *KpnI* and *SacI* (Fig. 2a, lane I). The 5.1-kb fragment in the *KpnI* digest probably represents the circle junction in unintegrated circular viral DNA containing two LTRs. Circular viral DNA molecules were observed for up to 2 weeks after infection of tissue culture cells; thus either circular viral DNA is stable for long periods of time or the cells in culture are reinfected continually.

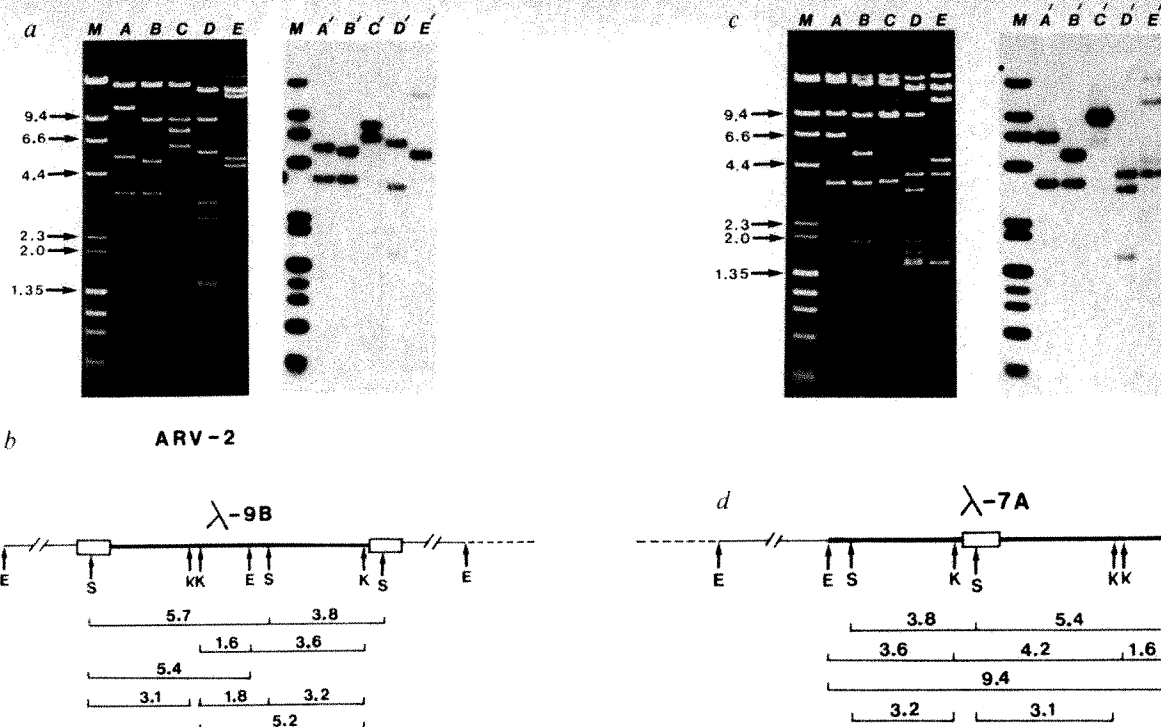


Fig. 3 Structures of recombinant phage containing ARV DNA. *a*, Purified λ -9B phage DNA digested by restriction enzymes; left-hand side, visualized with UV light; right-hand side, DNA transferred to nitrocellulose and annealed with viral cDNA probe. *M*, *Hind*III DNA fragments of phage λ and *Hae*III DNA fragments of phage Φ X174 (in kb). *A*, *A'*, *Sac*I digest; *B*, *B'*, *Eco*I and *Sac*I; *C*, *C'*, *Eco*I and *Sac*I; *D*, *D'*, *Kpn*I and *Eco*I; *E*, *E'*, *Kpn*I. *b*, Structures of recombinant phage DNA λ -9B. Heavy lines, viral DNA; open boxes, inferred LTRs; light lines, flanking-cell DNA; broken lines, phage vector DNA. *c*, As *a* but with λ -7A phage DNA. *d*, As *b* but with λ -7A phage DNA.

Methods: The DNA from HUT-78 cells infected with ARV-2 was treated with *Eco*RI under conditions yielding partial digest products (0.25 unit *Eco*RI per μ g cell DNA, 15 min, 37 °C) and centrifuged on 10–40% sucrose gradients (in buffer containing 0.5 M NaCl, 10 mM EDTA, 20 mM Tris-HCl, pH 7.5) at 35,000 r.p.m. for 12 h at 15 °C. Fractions were collected, a sample of each was electrophoresed on 1% agarose gels and the fractions containing 9–15-kb DNA were pooled. This size-selected DNA was concentrated by precipitation with ethanol and subsequently ligated to EMBL-4 bacteriophage DNA^{11,12} (digested with *Eco*RI, *Bam*HI and *Sal*I). (Packaging extract from Promega Biotech.) DP50 supF was the bacterial host. Plaquing efficiencies with native EMBL-4 vector DNA were about $1 \times 10^8 \mu\text{g}^{-1}$ and the background for the digested vector DNA alone was about $1 \times 10^5 \mu\text{g}^{-1}$. Plaquing efficiency for the ligated DNA (size-selected cell DNA and digested vector DNA) was $3 \times 10^6 \mu\text{g}^{-1}$. Ten 150-mm culture dishes were plated with 50,000 phage per culture, and phage were transferred to nitrocellulose in the double-life procedure¹¹. Viral cDNA probe (Fig. 2) identified recombinant phage plaques containing ARV DNA; these plaques were further purified and monitored with viral cDNA probe through secondary and tertiary platings. Plaque-purified phage containing ARV DNA were propagated in liquid culture in DP50; phage particles were collected and banded in CsCl gradients and recombinant phage DNA was prepared by phenol extraction followed by ethanol precipitation¹². We then digested 1 μ g purified phage DNA with restriction enzymes and electrophoresed them on 1% agarose gels.

To produce substrate for a recombinant DNA library, whole-cell DNA from cells infected with ARV-2 was partially digested with *Eco*RI; 9–15-kb cell DNA was cloned into an EMBL-4 bacteriophage λ vector and recombinant phage were identified with the virus-specific cDNA probe. Two examples of the 11 recombinant phage were selected for detailed description: λ -9B contained an insertion of full-length proviral DNA with flanking cell sequences and λ -7A contained a full-length viral genome permuted with respect to the *Eco*RI site. Viral DNA in each of these recombinant phage is 9.5 kb; the restriction endonuclease digest patterns are the same as those for viral DNA found in infected cells (Fig. 2). In addition to the two types of recombinant phage containing ARV DNA (Fig. 3), we obtained phage with the left half of the viral genome from the *Eco*RI site in viral DNA extending into flanking cell DNA and also phage with the right half of the viral genome from the *Eco*RI site in viral DNA extending into flanking cell DNA (our unpublished results).

To validate the cloned ARV DNA, a radioactive probe was prepared representing the entire cloned genome (see Fig. 4 legend). The cDNA probe (Figs 1, 2) and the probe representing cloned viral DNA anneal to the same DNA species in several restriction enzyme digests of DNA from infected cells (compare lanes *a*–*c* with *d*–*f* in Fig. 4).

We next assessed the relationship among three independent ARV isolates. Restriction enzyme digests of DNA from HUT-78 cells infected with ARV-3 and ARV-4 were analysed with the probe made from cloned ARV-2 DNA. The *Sac*I digests of ARV-3 DNA were similar to that of ARV-2 whereas the *Hind*III digests displayed different patterns. The *Sac*I digest and the *Pst*I digest of ARV-4 DNA differed from the corresponding digests of ARV-2 DNA (Fig. 4). The intensity of the annealing signals obtained with ARV-3 and ARV-4 samples was much lower (about 10-fold less) than that for ARV-2 DNA; this observation probably reflects the smaller number of cells infected in the ARV-3 and ARV-4 cultures or the smaller number of viral DNA molecules in individual cells. The virus-specific DNA fragments produced by *Sac*I treatment of ARV-3 and ARV-4 DNA totalled 9.0–9.5 kb, a value similar to that of ARV-2 and in agreement with the RNA genome size (Fig. 1). The antigenic relatedness of these three viral isolates was demonstrated in immunofluorescence studies with an AIDS reference serum (EW5111, from Dr Paul Feorino, Centers for Disease Control, Atlanta, Georgia). This serum reacted with HUT-78 cells infected with ARV-2 and human peripheral blood lymphocytes infected with ARV-3 and ARV-4 (ref. 4).

We have described here the characterization of the RNA and DNA genomes of ARV. Serological studies suggest that ARV,

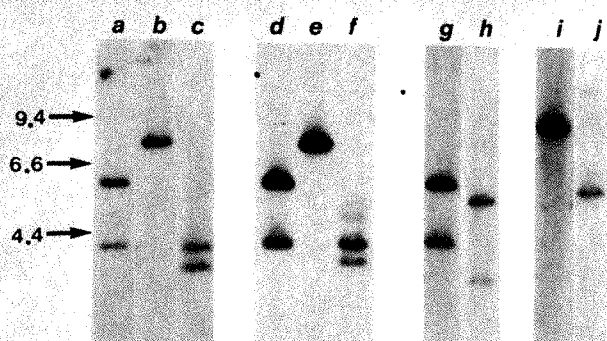


Fig. 4 Detection of ARV DNA in cells infected with three independent isolates of ARV. Whole-cell DNA was prepared from HUT-78 cell cultures infected with ARV-2, ARV-3 and ARV-4 and analysed by digestion with restriction enzymes as described in Fig. 3. DNA from recombinant phage λ -7A was digested with *EcoRI* and the 9.5-kb ARV DNA fragment was cloned into the *EcoRI* site of the vector pUC19. This recombinant plasmid was propagated, digested with *EcoRI* and the viral 9.5-kb DNA fragment was purified by electrophoresis in 1% agarose gels (low-melting agarose). Viral DNA was eluted from the agarose (Fig. 1), denatured by boiling in water for 2 min and used as template for reverse transcriptase with random DNA primers (calf thymus)⁶. Lanes a, b, c, DNA from HUT-78 cells infected with ARV-2 was digested with *SacI*, *PstI* and *HindIII*, respectively; viral cDNA probe was used (Fig. 2). Lanes d, e, f, DNA from HUT-78 cells infected with ARV-2 and digested with *SacI*, *PstI* and *HindIII*, respectively; probe to cloned ARV-2 DNA was used. Lanes g, h, DNA from HUT-78 cells infected with ARV-3, digested with *SacI* and *HindIII*, respectively, and annealed with probe to cloned ARV-2 DNA. Lanes i, j, DNA from HUT-78 cells infected with ARV-4, digested with *SacI* and *PstI*, respectively and annealed with probe to cloned ARV-2 DNA.

LAV and HTLV-III are related (our unpublished data; M. Bryant and M. Gardner, personal communication). DNA sequence analysis of these cloned ARV DNA molecules will reveal the genetic content of the virus and will provide a basis for comparisons with other viruses. Preliminary sequencing of ARV has shown small amounts of homology in portions of the gag and pol proteins of many retroviruses including Rous sarcoma virus, squirrel-monkey retrovirus and HTLV-I (our unpublished data). The significance of polymorphism of nucleotide sequences in independent isolates remains to be evaluated.

We thank our colleagues at Chiron and at the University of California, particularly Wendy Gee for assistance in the cloning, Andre Renard for optimizing virus growth conditions and purification procedures, Jim Stephens for help with tissue culture and Lacy Overby for help and advice. We also thank Dr David Kingsbury for making available the P3 containment facility at the Naval Biosciences Laboratory, Oakland, Pauline Montes and Dana Topping for preparation of typescripts and Tony Hoffman and Babak Banapur for their help with endogenous viral reactions. This work was supported in part by NIH grant CA-34980 and by a grant from the California State AIDS Task Force.

Received 24 September; accepted 20 November 1984.

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The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus

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Acquired immune deficiency syndrome (AIDS) is characterized by opportunistic infections and by 'opportunistic neoplasms' (for example, Kaposi's sarcoma)¹. Persistent generalized lymphadenopathy (PGL) is epidemiologically associated with AIDS, especially in male homosexuals. A subset of T lymphocytes positive for the CD4 antigen² (also termed T4 antigen), is depleted in AIDS and PGL patients. A retrovirus found in T-cell cultures from these patients³⁻⁵ is strongly implicated in the aetiology of AIDS because of the high frequency of isolation⁴ and the prevalence of specific antibodies⁶⁻⁸ in the patients. Here we have detected cell-surface receptors for the AIDS retrovirus (human T-cell leukaemia virus-III (HTLV-III) and lymphadenopathy-associated virus-1 (LAV-1) isolates) by testing the susceptibility of cells to infection with pseudotypes of vesicular stomatitis virus bearing retroviral envelope antigens, and by the formation of multinucleated syncytia on mixing virus-producing cells with receptor-bearing cells. Receptors were present only on cells expressing CD4 antigen; among 155 monoclonal antibodies tested, each of the 14 anti-CD4 antibodies inhibited formation of syncytia and blocked pseudotypes. Productive infection of CD4⁺ cells with HTLV-III or LAV-1 markedly reduced cell-surface expression of CD4. In contrast, receptors for HTLV-I and HTLV-II were not restricted to CD4⁺ cells, were not blocked by anti-CD4 antibodies; cells productively infected with HTLV-I and HTLV-II expressed surface CD4. Hence, we conclude that the CD4 antigen is an essential and specific component of the receptor for the causative agent of AIDS.

The first reported isolate of the AIDS retrovirus³ was named lymphadenopathy-associated virus as it was made from a PGL patient; two further isolates from AIDS patients in France were named immune deficiency-associated virus (IDAV-1, IDAV-2)^{9,10}, and American isolates⁴ have been collectively named HTLV-III. As the core proteins of the AIDS retroviruses isolated in France and America are closely related antigenically (M. G. Sarngadharan, R. C. Gallo and L. Montagnier, personal communication) and antisera from British AIDS and PGL patients specific for viral membrane antigens are cross-absorbed by virus from either source⁸, we consider LAV, IDAV and HTLV-III to be a single species of virus. Given the distant relationship of the AIDS virus to HTLV-I and HTLV-II (refs 11, 12) and as the term 'human T-lymphotropic virus' was originally coined for this family of retroviruses by Barré-Sinoussi *et al.*³, we shall use the general name, HTLV-III, for the AIDS retrovirus. The particular isolates used in the present study were LAV-1 (ref. 3) and HTLV-IIIb (ref. 4).

HTLV-I, HTLV-II and HTLV-III each show a tropism for CD4⁺ T cells¹³⁻¹⁵ although some non-T cells can be infected *in vitro*¹⁶⁻¹⁸. We have sought to characterize the cell-surface receptors for these viruses and to ascertain whether the tropism of the viruses is determined at the receptor level. As there are no quantitative infectivity assays for HTLV, we identified receptor-positive cells by assessing induction of multinucleated syncytia^{18,19}, and by the susceptibility of various cell types to pseudotypes of vesicular stomatitis virus (VSV) bearing envelope

Table 1 Receptors for HTLV subtypes

Type	Line	VSV pseudotypes of			
		HTLV-I	HTLV-II	HTLV-III	LAV-1
Human T cell	JM	+	+	+	+
Human T cell	CEM	+	+	+	+
Human T cell	CEM/LAV-1	+	+	-	-
Human sarcoma	HOS	+	+	-	-
Human sarcoma	HOS/HTLV-I	-	-	-	NT
Human carcinoma	HeLa	+	+	-	-
Cat kidney	CCC	+	+	-	-
Cat kidney	CCC/HTLV-I	-	-	-	NT

Pseudotypes of VSV (Indiana strain, wild type) were prepared as described previously²⁰ by adding excess neutralizing sheep anti-VSV serum to VSV collected from cells pre-infected with the HTLV strains. The pseudotype titres ranged between 10^4 and 10^5 PFU ml⁻¹. VSV(HTLV) pseudotypes were plated on the indicator cells in the presence of $2 \mu\text{g ml}^{-1}$ DEAE-dextran at $10^{3.5}$, $10^{2.5}$ and $10^{1.5}$ PFU per well. HOS, HeLa and CCC cells were infected as monolayers (10^6 cells per 30 mm-diameter well), whereas JM and CEM cells were infected in suspension. Monolayer cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS), and suspension cells were grown in RPMI 1640 + 10% FCS. CEM/LAV-1 were CEM cells productively infected with LAV-1 (ref. 8); HOS/HTLV-I and CCC/HTLV-I were productively infected with HTLV-I (refs 17, 20). After virus adsorption, the cells were washed and monolayer cells were overlaid with agar medium; suspension cells were plated in a layer of agar medium above a monolayer of 10^6 bovine MDBK cells (not susceptible to any strain of HTLV) to act as indicator cells for progeny VSV released from infected cells in suspension. Plaques were counted 2 or 3 days after plating of the pseudotypes. + Indicates 10^3 PFU ml⁻¹; - indicates 10 PFU ml⁻¹ for the cell types assayed; NT, not tested.

antigens of the different HTLV strains²⁰. When cells infected with retroviruses are superinfected with VSV, a proportion of the progeny VSV assemble sufficient envelope glycoprotein of the retrovirus to resist neutralization by hyperimmune anti-VSV serum²¹. The host range of such 'pseudotype' virions is restricted to cells expressing receptors specific to the retrovirus^{22,23}. Following penetration of the cell and uncoating of the virion, however, the transcribed VSV genome replicates to produce nonpseudotype particles which infect and destroy neighbouring cells. Thus, VSV(HTLV) pseudotypes provide a simple cytopathic plaque assay for HTLV receptors²⁰.

VSV pseudotypes were prepared with the envelopes of each HTLV species and their ability to form plaques was tested on different cells. Table 1 shows that VSV(HTLV-I) and VSV(HTLV-II) plate on a variety of cell types, whereas VSV(HTLV-III) is restricted to the two T-cell lines tested. CEM cells pre-infected with LAV-1 blocked pseudotypes of both LAV-1 and HTLV-III (the French and American isolates) but

not pseudotypes of HTLV-I and HTLV-II. HOS and CCC cells pre-infected with HTLV-I blocked pseudotypes of both HTLV-I and HTLV-II. These data indicate that HTLV-I and HTLV-II utilize the same receptor which is expressed on lymphoid, fibroblastic and epitheloid cell types, whereas HTLV-III uses a different receptor with a more restricted distribution.

We have shown previously^{19,20} that extensive cell fusion occurs when uninfected cells bearing HTLV receptors are mixed with cells producing HTLV-I or HTLV-II. As cells infected with HTLV-III have been observed to form multinucleated giant cells^{5,10}, we tested whether syncytia would be induced on mixing HTLV-III-producing HT/H9 cells⁵ with a 10-fold excess of uninfected CEM, JM or HT/H4 indicator cells. Many syncytia were observed with each cell type, commencing as early as 6 h after mixing and increasing in size and number until 48 h, when up to 50% of nuclei were contained within syncytia. No syncytia appeared after mixing HTLV-III-producing cells with HOS, HeLa or CCC cells, although these indicator cells formed abundant syncytia with HTLV-I and HTLV-II. Thus, syncytium induction can be used as an alternative to the pseudotype method for determining the presence of HTLV-III receptors on the surface of uninfected cells.

In a more extensive set of human cell lines, we used both syncytium induction and pseudotype infection to determine the presence of HTLV-III receptors; at the same time we determined the presence of the CD4 and CD5 antigens using monoclonal antibodies (CD5 is a 'pan-T' marker also called T1). Table 2 shows that only the cells expressing CD4 antigen were sensitive to syncytium induction by HTLV-III and infection by VSV(HTLV-III) pseudotypes. In particular, two non-T-cell lines, U937 and LCo-B, which expressed CD4 antigen but not the pan-T marker, expressed HTLV-III receptors. Thus, the CD4 antigen may be a component of the HTLV-III receptor.

Antibodies to antigens situated in or adjacent to the receptor site for HTLV-III might be expected to compete in binding to or obscuring the receptor, as has been shown for baboon endogenous virus²⁴. We therefore screened 155 monoclonal antibodies to T-cell surface antigens for their ability to inhibit the formation of syncytia induced by HTLV-III; these included 135 monoclonal antibodies from the 2nd International Workshop on Human Leukocyte Differentiation Antigens, which were screened without knowledge of their antigen specificity. Table 3 shows that all the monoclonal antibodies to CD4 antigen blocked cell fusion induced by HTLV-III. In addition, five other monoclonal antibodies (Leu8, two anti-MHC class II monoclonal antibodies and W80 and W151, for which the antigens

Table 2 Correlation of HTLV-III receptors with CD4 antigen expression

Indicator cells		HTLV-III receptor tests		% Cells immunofluorescent	
Origin	Designation	Syncytium induction	Pseudotype infection	Anti-CD4 (MT310)	Anti-CD5 (UCHT2)
T-cell leukaemia	JM	+++	+	100	100
T-cell leukaemia	CEM	+++	+	100	100
T-cell leukaemia	HT/H4	+++	NT	100	100
T-cell leukaemia	HT/H9	++	NT	90	90
T-cell leukaemia	MOLT-4	+++	+	100	90
T-cell leukaemia	HSB2	-	-	0	80
T-cell leukaemia	GH1	+	+	80	90
T-cell lymphoma (Sézary)	HUT 78	++	NT	30	70
HTLV-I-transformed T cell	CI10/MJ2	++	+	90	90
HTLV-II-transformed T cell	Ton-1	+	+	90	100
Burkitt's lymphoma (B cell)	Raji	-	-	0	0
B-lymphoblastoid (EBV)	LCo-B	+	-	30	0
Nine other B-lymphoblastoid lines	-	-	NT	0	0
Promyelocytic leukaemia	HL60	-	NT	0	0
Monocytic leukaemia	U937	++	+	70	0
Osteogenic sarcoma	HOS	-	-	0	0
Cervical carcinoma	HeLa	-	-	0	0

Twenty-five established human cell lines (listed above) were tested for expression of CD4 and CD5 antigens, for syncytial response to HTLV-III and for susceptibility to infection by VSV(HTLV-III) and VSV(LAV-1) pseudotypes.

have not been classified by the Workshop) weakly but consistently inhibited syncytium formation. To determine whether the antibodies were binding to indicator cells or to virus-producing cells, four anti-CD4 antibodies with syncytium-inhibiting properties were incubated separately with receptor-bearing cells and virus-producing cells, and the cells were mixed together after washing three times in antibody-free medium. In each case, strong inhibition of cell fusion was observed when antibody was preadsorbed to indicator cells but not when antibody was preadsorbed to virus-producing cells, indicating that the antibodies blocked syncytium formation by binding to cell receptor sites rather than to viral envelope antigens.

Monoclonal antibodies that were positive for syncytium inhibition, and an equivalent number of negative monoclonal antibodies selected from the different antigen groups, were tested for their ability to block VSV(HTLV-III) infection of CEM or GH1 cells, by incubating the cells with antibody before plating the pseudotype. Table 3 shows that all the anti-CD4 monoclonal antibodies, but not Leu8, W80 or W151, caused significant reductions (>80%) of pseudotype plaque-forming units (PFU). With some monoclonal antibodies, however, pseudotype inhibition was not always achieved, even at high concentrations; each

Table 3 Blocking of HTLV-III receptors by monoclonal antibodies

Antibody		No. of monoclonal antibodies positive/tested	
Group designation	Specificity	Syncytium inhibition	Pseudotype inhibition
CD1	Cortical thymocyte	0/9	NT
CD2	Pan-T, E receptor (T11)	0/16	0/1
CD3	Pan-T	0/13	0/2
CD5	Pan-T + B-CLL (T1)	0/11	0/4
CD6	Pan-T	0/7	NT
CD7	Pan-T	0/11	NT
CD4	Helper/inducer (T4)	14/14	14/14
CD8	Suppressor/cytotoxic (T8)	0/21	0/2
CD25	Interleukin-2 receptor (Tac)	0/9	0/1
CD26	Activation antigen p120	0/4	NT
—	Unclassified workshop monoclonal antibodies	2/16	0/3
—	Workshop positive and negative controls	0/11	0/3
—	T subset (Leu8)	1/1	0/1
—	T subset (S2)	0/1	0/1
—	MHC class I	0/1	0/1
—	MHC class II	2/8	0/5
—	β_2 -microglobulin	0/2	0/1

We tested 135 monoclonal antibodies supplied for the 2nd International Workshop on Human Leukocyte Differentiation Antigens for their ability to inhibit syncytium formation induced by HTLV-III_B; 20 further monoclonal antibodies were also tested. Two indicator cell lines, JM and HT/H4, tested separately for syncytium inhibition by each monoclonal antibody, both gave the same results. JM cells were positive for all the known CD antigen groups tested. Monoclonal antibodies were used at 1:20 dilutions in each test, and appearance of syncytia was read at 6 and 24 h after mixing HTLV-III-producing cells with indicator cells and monoclonal antibodies. In the first two experiments, all the monoclonal antibodies were coded and syncytia were independently scored by two authors, neither of whom knew the antigen specificity of the antibodies. Cultures showing >80% reduction of numbers of syncytia at 24 h were scored as positive. All the anti-CD4 monoclonal antibodies and 25 other monoclonal antibodies were also tested for pseudotype inhibition by treating indicator cells with $\sim 80 \mu\text{g ml}^{-1}$ antibody before adding VSV(HTLV-III_B) or VSV(LAV-1). For the pseudotype assays, 5×10^5 cells per 30-mm well of an adherent subline of CEM cells (and, in one experiment, adherent GH1 cells) were used as indicator cells. After virus adsorption, 10^6 HOS cells were added to the adherent T cells to form a dense layer for the enhancement of VSV plaque formation. Monoclonal antibodies giving >80% decrease in numbers of PFU were scored as inhibitory. Two or more independent experiments were done for each monoclonal antibody tested. The anti-CD4 antibodies were W68, W69, W70, W71, W75, W77, W78, W79, W107, W113, MT151, MT130, MT321 and Leu3a.

was tested in at least three separate experiments. Most monoclonal antibodies were tested at a 1:20 dilution, representing ~ 50 – $100 \mu\text{g}$ immunoglobulin per ml of medium. Four inhibitory anti-CD4 monoclonal antibodies with known immunoglobulin concentrations were titrated to determine the highest five-fold dilution inhibiting $\geq 80\%$ of VSV(HTLV-III) PFU. Two monoclonal antibodies (MT310 and MT321) had end-point titres of $16 \mu\text{g}$ immunoglobulin per ml, whereas Leu3a was positive at $3.2 \mu\text{g}$ immunoglobulin per ml and MT151 at $0.64 \mu\text{g}$ immunoglobulin per ml. The same monoclonal antibodies failed to inhibit the plating of VSV(HTLV-I) pseudotypes on CEM cells even at $100 \mu\text{g}$ immunoglobulin per ml.

The results of the blind screen of the antibodies from the 2nd International Workshop and the more extensive testing of selected monoclonal antibodies (Table 3) show that antibodies to CD4 antigen specifically prevent the HTLV-III envelope antigens from interacting functionally with the cell surface, presumably by binding to sites coincident with or adjacent to virus receptors. The significance of the three other monoclonal antibodies that partially blocked HTLV-III receptors is not yet clear.

Our experiments demonstrate that either pre-incubation with monoclonal antibodies to CD4 (Table 3) or pre-infection with the LAV-1 isolate of HTLV-III (Table 1) blocks the reception or penetration of VSV(HTLV-III) pseudotypes in CEM cells. We therefore tested whether productive infection of CD4-positive cells with HTLV-III alters the expression of the CD4 antigen at the cell surface. Table 4 summarizes the results of immunofluorescence assays performed on uninfected and HTLV-infected cells for CD4 antigen and for HTLV membrane antigens. We found a reciprocal relationship for expression of CD4 and HTLV-III antigens on the cell surface. In contrast, cells expressing membrane antigens of HTLV-I and HTLV-II also expressed CD4 antigen at a high level. Figure 1 shows the quantitation of anti-CD4 immunofluorescence by flow cytometry of three cell lines before and after HTLV-III infection. These results indicated that the amount of CD4 antigen available at the cell surface for monoclonal antibody binding was greatly reduced in HTLV-III-infected cells, whereas expression of major histocompatibility complex (MHC) class I (Fig. 1) and other membrane antigens (CD2, CD3 and CD25, data not shown) was unaffected.

Our results show that HTLV-III receptors are restricted to cells expressing CD4 antigen, suggesting that the CD4 antigen

Table 4 Reciprocal expression of HTLV and CD4 antigens on the cell surface

Lymphoid cell lines		Immunofluorescence	
Virus	Line	Anti-CD4	Anti-HTLV-III
Uninfected	H9	+++	—
	CEM	+++	—
	LCo-B	+	—
HTLV-III	H9/HTLV-III	±	+++
	H9/LAV-1	±	+++
	CEM/LAV-1	±	+++
	LCo-B/LAV-1	±	++
HTLV-I	C91/PL	+++	Anti-HTLV-I +++
	C10/MJ2	+++	++
HTLV-II	C12/18M	+++	Anti-HTLV-II +++
	Ton-1	+++	++

The cells were washed and treated without fixation with monoclonal MT151 (anti-CD4) followed by fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse IgG (Miles), or with sera from human patients specific for HTLV-I, HTLV-II or HTLV-III, followed by FITC-labelled goat anti-human IgG (Miles). Control human sera and control mouse immunoglobulin gave insignificant fluorescence. The results were scored by eye using a fluorescence microscope. 'Plus' marks indicate the intensity of fluorescence rather than the proportion of cells fluorescing.

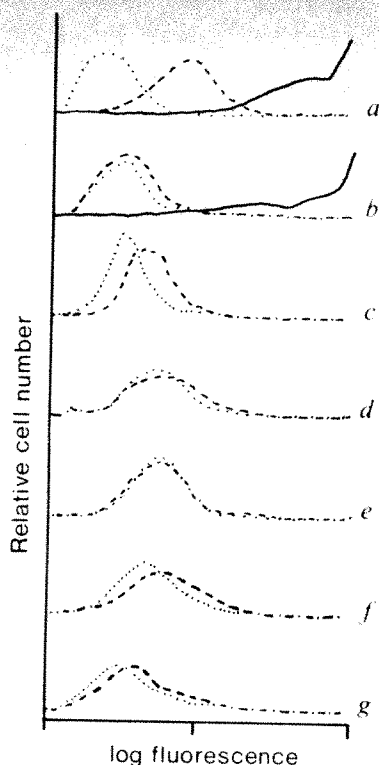


Fig. 1 Fluorescence histograms showing binding of monoclonal antibodies to uninfected and virus-infected cells. *a*, CEM; *b*, CEM/LAV-1; *c*, H9; *d*, H9/HTLV-III; *e*, H9/LAV-1; *f*, LCo-B; *g*, LCo-B/LAV-1. Solid lines, anti-MHC class I antibody; broken lines, anti-T4 antibody; dotted lines, control FITC-conjugated antibody. For fluorescence staining, 5×10^5 cells were incubated for 30 min on ice with 100 μ l of monoclonal antibody, washed once with 2 ml of cold medium (RPMI 1640 with 3% FCS and 0.02% sodium azide) and incubated for a further 30 min with 100 μ l of a saturating concentration of FITC-conjugated goat anti-mouse immunoglobulin (Sigma). After a further wash, the cells were fixed overnight in 0.5 ml of 3% formalin in phosphate-buffered saline with 1% FCS; 10,000 cells were analysed on a fluorescence-activated cell sorter fitted with a log amplifier. The anti-MHC class I antibody was W6/32; the anti-CD4 antibody was Leu3a. Controls comprised cells treated with FITC-labelled goat anti-mouse immunoglobulin alone.

itself is a necessary component of the receptor. Receptor blocking by monoclonal antibodies required high concentrations of antibody which were more effective in inhibiting formation of syncytia than in preventing infection by pseudotypes. Cell fusion induced by HTLV-III may require many more receptors than those required for the endocytosis of VSV(HTLV-III) particles, which may explain the relatively greater sensitivity to antibody of the syncytium inhibition assay.

In the accompanying paper, Klatzmann *et al.*²⁵ show that incubation of CD4⁺ cells in the presence of anti-CD4 antibodies, for the first 2 weeks of infection, inhibits the replication of HTLV-III (LAV-1), indicating that the CD4 antigen is required at some stage of the HTLV-III replication cycle. The diminution of CD4 expression on the surface of HTLV-III-infected cells (Table 4, Fig. 1) has also been observed by other investigators (refs 15, 25 and M. Popovic, personal communication); this finding may result simply from the viral envelope antigen competitively masking the binding sites for the monoclonal antibodies. Alternatively, the formation of a complex between the viral envelope glycoprotein and the CD4 receptor may result in a block to maturation, or rapid internalization, or capping and shedding of the CD4 antigen.

The CD4 antigen is a single-chain polypeptide of relative molecular mass 62,000², found on a T-cell subset which includes helper cells (for B-cell and macrophage responses), T cells which activate T suppressor cells and some cytotoxic T cells^{26,27}. There is evidence that when CD4⁺ T cells recognize antigen presented

by accessory cells, CD4 may interact with MHC class II antigens (for example, HLA-DR) expressed on the antigen-presenting cell^{28,29}. It is conceivable that the recognition of CD4 by the HTLV-III envelope antigen involves molecular mimicry of class II antigens; our preliminary data indicate that two out of eight monoclonal antibodies to class II antigens suppress formation of HTLV-III syncytia. Three other monoclonal antibodies (W80, W151 and Leu8) that are apparently not specific to CD4 antigen also blocked HTLV-III syncytia, but were less effective in blocking pseudotypes; these antibodies may recognize antigen-bearing molecules other than CD4 that also participate in HTLV-III reception and penetration, they may sterically hinder the presentation of CD4 to HTLV-III envelope antigen, or they may be required at a later stage in the process of cell fusion. It is noteworthy that the Leu8 antigen²⁶ is a marker for that subset of CD4⁺ cells that shows greatest depletion in AIDS and PGL³⁰.

The expression of CD4 antigen is not restricted entirely to subsets of T cells. Among the cells we studied (Table 2), the LCo-B lymphoblastoid line transformed by Epstein-Barr virus (EBV) is unusual in expressing CD4; nine other B-lymphoblastoid lines were negative for CD4 antigen and for HTLV-III receptors. The U937 monocytic cell line derived from a histiocytic lymphoma³¹ has been reported previously to express CD4 antigen as well as some normal cells of the monocyte-macrophage lineage^{32,33}. This raises the question of whether monocytes and macrophages may become infected by HTLV-III *in vivo*, as macrophage function is severely compromised in AIDS¹.

Most naturally-occurring, infectious transmitted retroviruses, such as avian, feline and bovine leukosis viruses, use receptors common to many cell types²³, as shown here (Table 1) for HTLV-I and HTLV-II. In contrast, HTLV-III recognizes a differentiation antigen. Some other viruses also appear to use receptors specific to the cell type most affected by infection, for example, the complement receptor type 2 for EBV³⁴, the acetylcholine receptor for rabies virus³⁵ and the β -adrenergic receptor for reovirus (B. Fields and M. Green, personal communication). AIDS and PGL are syndromes resulting primarily from a depletion of CD4⁺ Leu8⁺ cells in the peripheral blood and lymph nodes^{1,30}. Our finding that the CD4 antigen itself is an essential component of the receptor for the aetiological virus of AIDS helps to explain the nature of the disease. It remains to be seen whether receptor restriction is the sole determinant of cell tropism for HTLV-III.

We thank M. Exley for technical assistance; R. Cheingsong-Popov, A. J. Pinching and members of the Middlesex/University College Hospitals AIDS Research Group for helpful suggestions; M. Popovic, R. C. Gallo, L. Montagnier and J.-C. Chermann for supplying viruses and permissive cell lines; and participants of the 2nd International Workshop on Leukocyte Differentiation Antigens for monoclonal antibodies, in particular P. Rieber for the MT series. This study was supported by grants from the MRC and the Cancer Research Campaign.

Received 18 October; accepted 13 November 1984.

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T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV

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Many viruses, including retroviruses, are characterized by their specific cell tropism¹⁻³. Lymphadenopathy-associated virus (LAV) is a human lymphotropic retrovirus isolated from patients with acquired immune deficiency syndrome (AIDS) or related syndromes^{4,5}, that displays selective tropism for a subset of T lymphocytes defined by the expression of a surface glycoprotein of relative molecular mass 62,000 (62K) termed T4 (refs 6-8). This glycoprotein delineates a subset of T lymphocytes with mainly helper/inducer functions, while T lymphocytes of the reciprocal subset express a glycoprotein termed T8, have mainly cytotoxic/suppressor activities, and are unable to replicate LAV^{7,9}. Such a tropism may be controlled at the genomic level by regulatory sequences, as described for the human T-cell leukaemia viruses HTLV-I and -II (refs 2, 3). Alternatively or concomitantly, productive cell infection may be controlled at the membrane level, requiring the interaction of a specific cellular receptor with the virus envelope, as demonstrated recently for Epstein-Barr virus (EBV)¹. Therefore, we have investigated whether the T4 molecule itself is related to the receptor for LAV. We report here that preincubation of T4⁺ lymphocytes with three individual monoclonal antibodies directed at the T4 glycoprotein blocked cell infection by LAV. This blocking effect was specific, as other monoclonal antibodies—such as antibody to histocompatibility locus antigen (HLA) class II or anti-T-cell natural killer (TNK) target—directed at other surface structures strongly expressed on activated cultured T4⁺ cells, did not prevent LAV infection. Direct virus neutralization

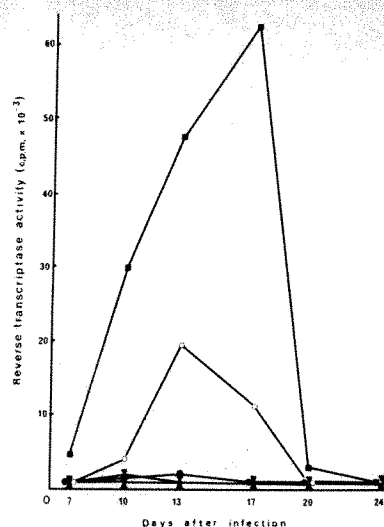


Fig. 1 Monoclonal anti-T4 antibodies, but not anti-HLA class II or anti-TNK-target, inhibit replication of LAV in T4⁺ cultured cells. ■, Anti-HLA class II monoclonal antibodies (mixture of CA2.06, CA1.41 and BM50); ○, anti-TNK-target antibody; ●, 12T4 anti-T4 antibody; ▼, OKT4 anti-T4 antibody; ▲, 19Thy anti-T4 antibody. Cell cultures and determinations of reverse transcriptase activity were performed as described in Table 1.

by monoclonal antibodies was also ruled out. These results strongly support the view that a surface molecule directly involved in cellular functions acts as, or is related to, the receptor for a human retrovirus.

While studying the LAV selective tropism, we observed that, at the time of virus replication, a decreased proportion of purified cultured LAV-infected T4⁺ lymphocytes was labelled by immunofluorescence staining with anti-T4 monoclonal antibodies (Table 1)⁷. As at that time the remaining viable cells retained their specific T-cell marker T3 (ref. 9), we assumed that this phenomenon could not be related to the cytopathic effect of LAV⁷, but was probably due to either modulation of T4 molecules at the cell membrane or steric hindrance of antibody-binding sites. Both cases imply interaction of viral particles produced in the culture with the expression of T4. Consequently, we designed experiments to assess whether the T4⁺ tropism of LAV was directly related to the presence of this glycoprotein on T4⁺ lymphocytes.

Three antibodies (OKT4, Coulter clone 12T4D11 and DFCI clone 19 Thy 5D7) specific for distinct epitopes of the T4 glycoprotein were used in these studies. As controls we used four different monoclonal antibodies, three directed against HLA class II gene products, mainly the DR molecule (given by D. Charron)¹⁰, and anti-TNK-target, which recognizes a 140K surface molecule strongly expressed on all cultured T lymphocytes¹¹. T4⁺ lymphocytes (stimulated with phytohaemagglutinin (PHA) for 3 days) were obtained from peripheral blood mononuclear cells of normal donors as described previously^{6,7}.

Table 1 Reduced expression of T4 molecule in LAV-infected T4⁺ lymphocytes

Cell fraction	LAV	% Lymphocytes stained					
		Before the peak of RT (day 4)			After the peak of RT (day 11)		
		OKT4	OKT4A	OKT8	OKT4	OKT4A	OKT8
T4 ⁺	—	95	94	3	78	76	1
T4 ⁺	+	85	89	1	21	25	3
T8 ⁺	—	1	NT	95	NT	NT	94
T8 ⁺	+	1	NT	85	NT	NT	87

• Purified T4⁺ and T8⁺ lymphocytes were obtained by cellular affinity chromatography as described previously^{6,7} and cultured in the presence of 1% PHA-M in RPMI1640 medium supplemented with 10% fetal calf serum. After 3 days, 5×10^6 cells were incubated for 30 min at 37 °C with 50,000 c.p.m. of LAV reverse transcriptase-containing supernatant. After washing, cells were resuspended in 5 ml of culture medium containing IL-2, anti-γ-interferon serum and polybrene⁴. Reverse transcriptase activity (RT) was determined twice weekly in cell-free supernatants as reported elsewhere⁴. In this experiment, RT peaked at day 8, having reached 1×10^5 c.p.m. Indirect immunofluorescence staining was performed as described previously using the OKT4, OKT4A and OKT8 monoclonal antibodies (Ortho-Diagnostics System)⁶.

Table 2 Monoclonal antibodies inhibit infection of lymphocytes by LAV

Antibody	Reverse transcriptase activity (c.p.m. $\times 10^{-3}$)	Lymphocyte concentration ($\times 10^6$ ml $^{-1}$)
-	39	1.3
Anti-T4	2	1.2
Anti-class II	54	1.4

Purified T4⁺ lymphocytes were obtained and cultured as described for Table 1. After 3 days, cells were centrifuged and 5×10^6 cells incubated with 500 μ l of culture medium containing saturating concentrations (1:200) of the respective antibodies or medium alone as control. After 20 min at room temperature, the cells were washed once and incubated for 30 min at 37 °C with 50,000 c.p.m. of LAV reverse transcriptase-containing supernatant. After washing, cells were resuspended in 5 ml of culture medium to which the respective monoclonal antibody had been added at a 1:400 dilution. Reverse transcriptase activity was determined twice weekly for cell-free supernatants obtained at the time when cultures were adjusted to 1×10^6 cells per ml and new medium added⁴. The results shown were obtained at the peak of virus production in this experiment (day 6 after infection). Lymphocyte concentrations represent cell growth from day 3 (when the number of cells was adjusted to 1×10^6 ml $^{-1}$) to day 6.

Table 3 Incubation with monoclonal antibodies does not neutralize LAV

LAV	Antibody	Reverse transcriptase activity (c.p.m. $\times 10^{-3}$)
-	-	1
+	-	32
+	T3	35
+	T4	31
+	T8	30

LAV-containing supernatant was incubated for 30 min at 37 °C with either OKT3, OKT4 or OKT8 (Ortho-Diagnostic) monoclonal antibody at the concentrations used in the previous experiments; 2 ml of this supernatant were then mixed with 1 ml of goat anti-mouse immunoglobulin-coated Sepharose 6-MB beads. After incubation of this mixture for 30 min at room temperature, reverse transcriptase activity was measured in 2 ml of the supernatant.

and incubated for 20 min at room temperature with saturating concentrations of antibodies. After washing, cells were incubated with LAV-containing supernatant for 30 min at 37 °C, using 10,000 c.p.m. of reverse transcriptase activity per 10^6 cells⁴. Unadsorbed virus was then washed out and cells were cultured in medium containing anti-interferon serum, interleukin-2 (IL-2)⁴ and the respective monoclonal antibodies. As infectious particles can persist in culture medium even after washing and further infect lymphocytes that are no longer coated by antibodies shed in the medium, we added antibodies to the culture for the first 2 weeks to prevent such late infection.

Incubation and culture of T4⁺ cells with anti-TNK-target or anti-HLA class II monoclonal antibodies did not prevent virus production, as detected by reverse transcriptase activity in the culture supernatants as well as by the appearance of a cytopathic effect⁷. In contrast, incubation with either of the anti-T4 antibodies completely inhibited virus replication in these cells (Table 2, Fig. 1); we could detect no reverse transcriptase activity or cytopathic effect for as long as 1 month after virus infection, even after discontinuing the addition of anti-T4 antibody to culture medium on day 13 (Fig. 1). Such results could not be attributed to the decreased proliferation of cultured cells in the presence of these monoclonal antibodies (Table 1; ref. 12). Neutralization of LAV particles by anti-T4 antibody was ruled out. Viruses were not retained by the goat anti-mouse immunoglobulin-coated Sepharose 6-MB beads which were used to enrich the T4⁺ population^{6,7} after incubation with these

antibodies, at the ratios and concentrations used in the previous experiments (Table 3). Nonspecific blockade of virus penetration by modification of the membrane properties after antibody binding was also unlikely, as other monoclonal antibodies which bind to glycoproteins expressed in high amounts on the membranes of activated cells did not prevent viral infection.

In the conditions used, the amount of reverse transcriptase activity appeared to vary according to the antibody used. The possibility that some of these antibodies could even enhance virus penetration or production (Table 2) is being investigated.

Thus, these results strongly suggest that LAV tropism depends on the interaction of LAV with the T4 molecule; they are in accord with the data presented in the accompanying paper¹³, which show the relationship between the T4 expression of different lymphoid cell lines and their susceptibility to LAV and HTLV-III, a similar retrovirus isolated from AIDS patients¹⁴. Replication of LAV in EBV-transformed lymphoblastoid cell lines¹⁵ could indeed be associated with a significant T4 expression on these cells¹³.

The present findings strongly suggest that the T4 glycoprotein is at least associated with all or part of the receptor for LAV, of particular interest in view of the involvement of this molecule in human T-cell functions. Indeed, this glycoprotein delineates a subset of lymphocytes which primarily mediate induction of the immune response^{8,9}. Furthermore, recent studies have suggested that T4 is involved in the recognition of HLA class II gene products^{16,17}. Taken together, these data provide a molecular basis for the previously described tropism of LAV for the inducer lymphocyte subset. In addition, they may more generally reflect one of the mechanisms of interaction between retroviruses and immunocompetent cells. Finally, these results should help to delineate future therapeutic strategies in the treatment of AIDS.

We thank Drs D. Charron, E. L. Reinherz, S. F. Schlossman, Ortho-Diagnostic System and Coulter-Immunology for providing the different monoclonal antibodies; Mrs E. Brisson and F. Chapuis for technical assistance; and Mrs C. Debrun for secretarial help. This work was supported by the Conseil Scientifique de l'UER Pitié-Salpêtrière. T.H. is a fellow of the Leukemia Society of America.

Received 18 October; accepted 9 November 1984.

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Cloning, sequence and expression of human interleukin-2 receptor

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T lymphocytes, essential for the generation of a normal immune response, require the presence of the lymphokine interleukin-2 (IL-2) in order to proliferate^{1,2}. Cells that respond to IL-2 possess a surface receptor glycoprotein specific for this lymphokine^{3,4}. We have recently purified and chemically characterized the IL-2 recep-

tor from both phytohaemagglutinin-activated human T cells and the human T-cell lymphoma HUT-102 (ref. 5). From the NH₂-terminal protein sequence obtained in that study, we have now used synthetic oligonucleotides to probe a complementary DNA library, prepared from HUT-102 messenger RNA, for the presence of cDNA clones that might code for the IL-2 receptor. Two cDNA clones were isolated which had closely related DNA sequences. Interestingly, only one coded for an active receptor when transfected into COS-7 cells. This clone contained a 216-base pair (bp) insert that was not present in the other clone. The insert was flanked by an 8-bp direct repeat reminiscent of a transposable element, and appeared to code for a region of marked structural homology to the NH₂-terminal region of the receptor molecule.

Polyadenylated mRNA from HUT-102 cells was purified and used to construct a cDNA library by standard techniques (Figs 1, 2), and oligonucleotide probes corresponding to the amino acid sequence of the IL-2 receptor (Cys-Asp-Asp-Asp-Pro-Pro)⁵ were synthesized. As there were 64 possible 17-nucleotide sequences that could encode these amino acids, we carried out primer extension experiments to define a more precise probe (Fig. 1). Of the six oligonucleotide pools tested, only pool N (5'-GGG²GGGTCGTCGTCACA-3') gave clear primer extension products, of 230 and 280 nucleotides. This pool was therefore used as a probe to screen the HUT-102 cDNA library (Fig. 2).

Two cDNA clones, designated pN1 and pN4, having insert sizes of 1.0 and 2.8 kilobase pairs (kb), respectively, were selected for further analysis. Restriction mapping and cross-hybridization experiments showed that these two clones were closely related, but not identical (Fig. 2a). pN4, in addition to being considerably larger than pN1, contained an insert of about 200 bp not present in pN1. To establish the relationship between these cDNA clones and their possible identity to the IL-2 receptor sequence, we determined their nucleotide sequence (Fig. 2b). Both clones contained the sequence of the oligonucleotide probe N, with a single base pair mismatch, and encoded the amino acid sequence determined for the NH₂-terminus of the IL-2 receptor⁵. They also encoded a stretch of 21 amino acids immediately preceding the NH₂-terminal sequence, starting with methionine and showing many of the characteristics of a hydrophobic signal peptide expected for membrane or secreted proteins.

Both pN1 and pN4 had a single large open reading frame, of 200 and 272 amino acids, respectively, terminating at the same position (nucleotide 861). However, pN4 contained 216 nucleotides not found in pN1 (nucleotides 370-585). This insert was bounded by direct repeats of the 8-bp sequence TTCCAGGT, which was found only once in pN1. Following this insert, the same reading frame was maintained in pN4 and pN1. Only four nucleotide differences were found between the two sequences. These were clustered towards the NH₂-terminal portion of the protein, and were predicted to cause three amino acid replacements, Glu 29 to Lys, Lys 87 to Glu, and Ile 88 to Met. A search of the data bases of the National Biomedical Research Foundation, Genbank and the European Molecular Biology Laboratory detected no significant homologies with other sequences.

To determine whether one or both cDNA clones could encode a functional IL-2 receptor, we attempted their expression in COS-7 monkey kidney cells (Fig. 3). Two plasmids, pN1/N4-S and pN1/N4-X, containing the coding regions of pN4 and pN1, respectively, were transfected into COS-7 cells. The cells were collected 48 h later and assayed for expression of the IL-2 receptor by their ability to bind either ¹²⁵I-labelled IL-2 or 2A3, a monoclonal antibody directed against the IL-2 receptor⁵. We found (Fig. 3b, c) that COS-7 cells transfected with pN1/N4-S could bind specifically both IL-2 and the monoclonal anti-IL-2 receptor antibody, but that neither pN1/N4-X nor mock-transfected COS-7 cells showed any specific binding of either IL-2 or antibody. As pN1/N4-S contains the pN4 coding region, this result indicated that pN4 encodes a functional IL-2 receptor protein, while pN1 does not. The receptor in transfected COS-7 cells migrated on SDS-polyacrylamide gels with a relative

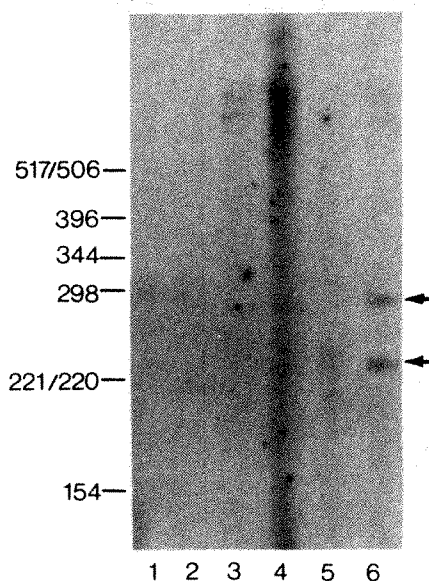


Fig. 1 Primer extension analysis of T-cell RNA. The autoradiogram shows:

lane 1, hybridization with the oligonucleotide pool I, 5'-GGGGG²TCATCATCACA-3';
lane 2, pool J, GGAGG²TCATCATCACA;
lane 3, pool K, GGTGG²TCATCATCACA;
lane 4, pool L, GGCGG²TCATCATCACA;
lane 5, pool M, GG²GGGTCGTCGTCACA;
lane 6, pool N, GG²GGGTCGTCGTCACA.

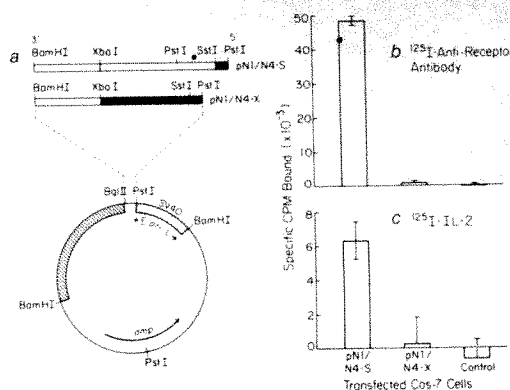
M, markers are ³²P-labelled *Hinf*I fragments of pBR322. In other experiments (data not shown), pool N gave the same primer extension products with RNA from HUT-102 cells, but not with RNA from IL-2 receptor-negative HL60 cells, and was therefore a good candidate for an IL-2 receptor-specific probe.

Methods: Peripheral blood T cells were isolated as described previously¹³ and stimulated for 20 h with the mitogens concanavalin A (20 µg ml⁻¹) and phorbol myristate acetate (10 ng ml⁻¹). Total RNA was isolated by the guanidinium thiocyanate/caesium chloride method¹⁴. Oligonucleotides were ³²P-labelled to a specific activity of 10⁶ c.p.m. per ng with T4 polynucleotide kinase and [γ -³²P]ATP (7,000 Ci mmol⁻¹, NEN). Each probe (5 × 10⁵ c.p.m.) was co-precipitated with 20 µg of RNA, redissolved in 5.5 µl 10 mM Tris pH 8.3, 1 mM EDTA, 4.5 µl 2 M KCl, heated at 65 °C for 5 min, then hybridized at 50 °C for 1 h. The reaction was adjusted to 50 mM Tris pH 8.3, 140 mM KCl, 10 mM MgCl₂, 5 mM dithiothreitol, 100 µg ml⁻¹ actinomycin D, 100 µg ml⁻¹ bovine serum albumin (BSA) and 10 U reverse transcriptase in a final volume of 60 µl. This was incubated at 42 °C for 1 h, followed by the addition of EDTA to 20 mM final concentration. Following phenol:chloroform (1:1) extraction and ethanol precipitation, the nucleic acids were redissolved in 80% formamide, 100 mM Tris-borate pH 8.3, 1 mM EDTA and electrophoresed on a 4% polyacrylamide/8 M urea gel. Following electrophoresis, the gel was dried and exposed to X-ray film.

molecular mass (*M_r*) of 50,000, as detected by Western blot analysis using ¹²⁵I-labelled 2A3 (data not shown).

To study the expression of IL-2 receptor mRNA, we probed Northern blots of RNA from HUT-102 cells and activated peripheral blood T cells (Fig. 4). A probe derived from the *Sst*I-*Bam*HI fragment of pN4 hybridized to two classes of RNA of about 1.4 and 3.4 kb, present in both HUT-102 and T-cell RNA (Fig. 4a). These RNA species were present at less than 1% of these levels in resting T cells (data not shown). Interestingly, a probe specific for the insert in pN4, consisting of the 240-bp *Pst*I-*Bgl*II fragment, hybridized to both RNA classes in HUT-102 and T-cell RNAs (Fig. 4b).

The relationship between pN1 and pN4 remains unknown. pN1 could represent a cloning artefact or an aberrant transcript specific to HUT-102 cells. If pN1 indeed represents a *bona fide* mRNA species, it could be the product of a gene at a locus distinct from, or allelic to, that of pN4. Alternatively, one gene could give rise to both classes of transcripts via alternative RNA



Methods: a, Double-stranded cDNA was synthesized from HUT-102 polyadenylated RNA by standard procedures¹⁴. The cDNA was size fractionated on a Sephacryl S-400 column and fractions > 500 bp in length were pooled and tailed with dCMP residues¹⁵. The tailed cDNA was annealed to dGMP-tailed, *Pst*I-cut pBR322 (NEN) and used to transform *Escherichia coli* strain MM294 according to the procedure of Hanahan¹⁶. Approximately 2×10^6 transformants were obtained from 100 ng of cDNA. Pools of 5,000 transformants were used to make small-scale plasmid DNA preparations¹⁷, digested with *Pvu*II and *Hind*III, electrophoresed on a 0.8% agarose gel, blotted to nitrocellulose filters and hybridized to ³²P-labelled oligonucleotide pool N (10^6 c.p.m. ml^{-1} in $6 \times \text{SSC}$, 0.5% NP40, 0.1% sarcosyl, $5 \times \text{Denhardt's}$ solution containing $100 \mu\text{g ml}^{-1}$ salmon sperm DNA at 50°C for 16 h). Blots were washed extensively in $6 \times \text{SSC}$ at room temperature and then for 5 min at 55°C before autoradiography. Positive pools were chosen for colony hybridization¹⁸ experiments using the same conditions. Identified transformants which hybridized strongly with the probe were purified. Restriction maps were generated using standard techniques¹⁴. **b,** Plasmids pN1 and pN4 were digested with *Pst*I, *Sst*I, *Xba*I and *Bam*HI in various combinations and cloned with M13mp18 or M13mp19 (ref. 19). DNA sequencing was done by the chain termination method²⁰ as described previously^{21,22}.

Methods: **a,** Plasmids were transfected into COS-7 cells as described previously.^{28,29} Monolayers of COS-7 cells (10^6 cells per 10-cm plate) were washed twice with Tris-buffered saline (TBS) and exposed to $10\ \mu\text{g}$ of DNA per plate in 1 ml TBS containing $500\ \mu\text{g}\ \text{ml}^{-1}$ DEAE-dextran ($M_r\ 5 \times 10^5$, Sigma) for 30 min at room temperature. The cells were washed once more with TBS and fed with growth medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum) containing $100\ \mu\text{M}$ chloroquine. After 5 h at 37°C , the medium was replaced by growth medium without chloroquine, and the cells were incubated at 37°C for 48 h, then collected by scraping. **b,** c , ^{125}I -labelled 2A3 (specific activity 9.8×10^{14} c.p.m. mmol^{-1}) was purified and radiolabelled as described previously.² IL-2 was purified by two-step reverse-phase HPLC³⁰ and radiolabelled using the Enzymobead radioiodination reagent (BioRad) essentially following the manufacturer's specifications. Briefly, $50\text{-}\mu\text{l}$ aliquots of IL-2 (5×10^6 U) in acetonitrile and trifluoroacetic acid were combined with $50\ \mu\text{l}$ of $0.2\ \text{M}$ sodium phosphate pH 7.2, and the acetonitrile was evaporated under nitrogen. Then $50\ \mu\text{l}$ of Enzymobead reagent, $10\ \mu\text{l}$ of ^{125}I (1 mCi) and $10\ \mu\text{g}$ of 2.5% β -D-glucose were added and the mixture was incubated at 25°C for 10 min. Sodium azide ($20\ \mu\text{l}$ of $25\ \text{mM}$) and sodium metabisulphite ($10\ \mu\text{l}$ of $5\ \text{mg}\ \text{ml}^{-1}$) were then added and after 5 min at 25°C , iodinated IL-2 was separated from unbound ^{125}I by chromatography on a 2-ml Sephadex G-25 column equilibrated in $0.05\ \text{M}$ sodium phosphate, pH 7.2, containing 0.1% BSA. Based on an initial biological specific activity for IL-2 of 1×10^6 U per μg protein, the radiolabelled preparation had an estimated specific activity of 1×10^{15} c.p.m. mmol^{-1} . Binding assays were performed as described previously.³¹ Cells (1.2×10^6) were incubated with either $5 \times 10^{-9}\ \text{M}$ ^{125}I -2A3 or $1.3 \times 10^{-8}\ \text{M}$ ^{125}I -IL-2 in a final volume of $150\ \mu\text{l}$ for 30 min at 37°C . Nonspecific binding was measured in the presence of a 1,000-fold molar excess of unlabelled 2A3 or a 150-fold molar excess of unlabelled IL-2. Replicate $70\text{-}\mu\text{l}$ aliquots of incubation mixtures were centrifuged through phthalate oil, and **b** and **c** show specific c.p.m. bound to separated cell pellets.

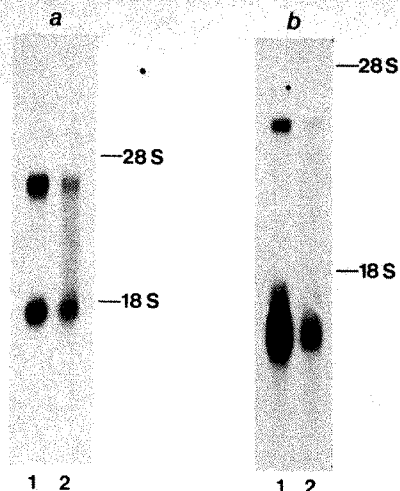


Fig. 4 Northern blot analysis of RNA from HUT-102 and mitogen-stimulated peripheral blood T cells. Poly(A)⁺ mRNA was purified³² from whole-cell RNA, isolated as described in Fig. 1 legend, electrophoresed on 1.5% agarose gels containing formaldehyde and transferred to nitrocellulose filters as described elsewhere¹⁴. Blots were hybridized with ³²P-RNA probes, synthesized by SP6 polymerase as described previously³³. Restriction fragments to be used for probes were subcloned into the pSP64 vector (Promega Biotec). **a**, Probe from the *Sst*I-*Bam*HI fragment of pN4. **b**, Probe from the pN4-specific 240-bp *Pst*I-*Bgl*II fragment of pN4 (Fig. 2). Hybridization was for 16 h at 63 °C in Stark's buffer³⁴. Blots were washed at 68 °C in 0.1 × SSC for 1 h and autoradiographed for 4 h with intensifying screens. Lane 1, 2 μg of poly(A)⁺ whole-cell RNA from HUT-102; lane 2, 2 μg of poly(A)⁺ whole-cell RNA from peripheral blood T cells stimulated as in Fig. 1. The position of large (28S) and small (18S) ribosomal RNAs observed by ultraviolet shadowing of the gel before blotting are indicated.

splicing and/or polyadenylation mechanisms. We are currently attempting to resolve these issues by isolating additional cDNA and genomic clones of the IL-2 receptor gene.

Figure 2b shows the sequence coding for a protein of *M_r* 28,428 and defines three structural regions. Region 1 covers residues 1–102, terminating with the first 8-bp repeat. It is potentially rich in carbohydrate, with possible *N*-linked glycosylation sites at Asn residues 49 and 68. *O*-linked glycosylation sites are difficult to predict, but serine- and threonine-rich areas are clustered in the same region around residues 50–54 and 62–66. The *M_r* of the receptor isolated from HUT-102 cells ranges from 47,000 (estimated from the specific activity of the purified receptor of 21,200 fmol of receptor per μg protein⁵) to 55,000 (estimated from the migration of the protein on SDS-polyacrylamide gels⁵). Similar sizes have been reported in studies of radiolabelled receptor^{6,9}. Results of pulse-chase experiments and studies using tunicamycin and glycosidase indicate a protein of *M_r* 33,000–40,000 that is a precursor to the mature protein^{6,9}. Our results suggest that the receptor could easily be composed of 50% carbohydrate.

Region 2 is present only in pN4 and covers residues 103–174, terminating with the second 8-bp repeat. It has a striking homology to region 1 in that if the Cys residue at position 104 is aligned with the Cys at position 3, all of the residues boxed in Fig. 2b, including all of the Cys residues, are identical between the two regions. A hydrophilicity plot of the amino acid sequence¹⁰ revealed that this region contains a stretch of 18 largely hydrophobic amino acids (residues 118–135). Because region 2 is absent from pN1 and as pN1/N4-X fails to encode a detectable receptor in transfected COS-7 cells, it is tempting to speculate that this region is responsible for the IL-2 and 2A3 binding properties of the protein encoded by pN4 (pN1/N4-S). It is interesting that the sequence encoding region 2 of pN4 is flanked by 8-bp direct repeats. The generation of small direct repeats enclosing an insertion is characteristic of many types of transposable element¹¹. However, it is unusual for a transposable

element to encode a protein domain that is essential for the function of that protein. We have no evidence that the protein encoded by pN4 is sufficient by itself to deliver a transmembrane signal. It therefore remains possible that it is the IL-2 binding component of a larger IL-2 receptor complex, as previously suggested¹².

The third region consists of the 77 residues extending from the second 8-bp repeat to the C-terminus of the protein and is present in both pN1 and pN4. It contains a presumptive membrane-spanning region of 23 hydrophobic residues between positions 218 and 241, followed by a short, highly basic C-terminal peptide.

The availability of a cDNA encoding the IL-2 receptor should help to elucidate the regulation and function of a protein that is intimately involved in growth control of human T cells.

We thank Toni Read for the preparation of the manuscript, Kate McKereghan, Catherine Grubin, Steve Kosowski, Steve Gimpel, Tami Parr, Susan Call, Della Friend and Andrew Lewis for technical assistance, Ken Grabstein for help in the preparation of T-cell RNA and Tom Hopp for performing the hydrophilicity analysis.

Received 28 September; accepted 13 November 1984.

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Primary structure of human T-cell receptor α-chain

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The T-cell receptor has been studied intensely over the past 10 years in an effort to understand the molecular basis for major histocompatibility complex (MHC) restricted antigen recognition^{1–9}. The use of anti-receptor monoclonal antibodies to isolate and characterize the receptor from human and murine T-cell clones^{10–15} has shown that the protein consists of two disulphide-linked glycopeptides, α and β, distinct from known

immunoglobulin light and heavy chains. Like immunoglobulin light and heavy chains, however, both the α - and β -chains are composed of variable and constant regions¹⁶⁻¹⁸. Molecular cloning has revealed¹⁹⁻²³ that the β -chain is evolutionarily related to immunoglobulins, and is encoded in separate *V* (variable), *D* (diversity), *J* (joining) and *C* (constant) segments that are rearranged in T cells to produce a functional gene^{19-21,24-27}. We report here cDNA clones encoding the α -chain of the receptor of the human T-cell leukaemia line HPB-MLT. Using these cDNA probes, we find that expression of α -chain mRNA and rearrangement of an α -chain *V*-gene segment occur only in T cells. The protein sequence predicted by these cDNAs is homologous to T-cell receptor β -chains and to immunoglobulin heavy and light chains, particularly in the *V* and *J* segments.

The HPB-MLT α -chain peptides, the corresponding oligonucleotide families and the method used for isolating and subcloning the two α -chain cDNA clones, pGA-1 and pGA-5, are described in detail in Fig. 1 legend. The 570-base pair (bp) *EcoRI*-*PstI* fragment of the pGA-1 insert corresponding to the *V*, *J*, and part of *C* of the α -chain (see Fig. 3) was used to investigate the expression of α -chain mRNA. The fragment was labelled by nick-translation and used to probe mRNA from several T-cell and non-T-cell tumour lines (Fig. 2a). A mRNA species of 1.5 kilobases (kb) was detected in HPB-MLT and in a second T-cell tumour, JURKAT. No mRNA species were detected in the erythroleukaemia line, K562, or in the two B-cell tumour lines U266 and Daudi. Thus the presumptive α -chain mRNA is T-cell-specific and 1.5 kb in length.

The organization of β -chain and immunoglobulin genes coupled with the partial α -chain amino acid sequence²³ predicted that the α -chain should be encoded in gene segments undergoing somatic rearrangements in T cells. Southern blot analysis probing with the 570-bp *V*-*J*-*C* fragment (Fig. 2b) demonstrated the existence of T-cell-specific DNA rearrangements in HPB-MLT and JURKAT but not in two other T-cell lines, CEM and F35.3, or in any of the three non-T-cell lines examined. To determine which portion of the α -chain was encoded on the rearranging fragments in HPB-MLT and JURKAT, we repeated the analysis with separate *V*- and *C*-region probes (Fig. 2b). The *C*-region probe identified a 0.6-kb *PvuII* germ-line fragment and a 5.0-kb *BamHI* germ-line fragment which were not rearranged in any cell line we examined. Similar results were obtained using DNA digested with either *HindIII* or *EcoRI*, indicating that these restriction sites are present in the intron between the *J_α* gene segments and the *C_α* gene segment. The *V*-region probe detected rearrangements in HPB-MLT and JURKAT, but not in any other cell line, suggesting that HPB-MLT and JURKAT have rearranged the same *V_α* gene segment. However, the fact that the rearranged fragments differed in size by ~2 kb may imply a rearrangement to a different *J_α* in HPB-MLT and JURKAT. The detection of a single germ-line fragment with this *V_α* indicates that it is not a member of a large cross-hybridizing *V_α* family such as those often seen among immunoglobulin *V* genes. The failure to detect rearrangement of this *V_α* in CEM or F35.3 is not surprising since not all T cells are expected to rearrange and express the same *V* gene. Taken together, these data indicate that, as with other immunoglobulin-like molecules, the α -chain is encoded by separate, non-contiguous segments which somatically rearrange in T cells to produce a functional gene.

The nucleotide sequences of the inserts in pGA-1 and pGA-5 were determined and the portions corresponding to α -chain mRNA deduced (Fig. 3). The two sequences confirmed each other. The α -chain nucleotide sequence and its predicted translated protein (Fig. 3b) shows that of the eight α -chain tryptic peptide sequences which we previously reported²³, seven are present in the predicted protein sequence (A-G), as well as a ninth peptide (I), sequenced but not previously reported. One of the previously-reported peptides (H) is not present. Presumably this peptide was a high yield product of a minor contaminant in our α -chain protein preparation.

As the initiation ATG codon was apparently lost during the

preparation and cloning of these cDNAs, the starting point of the coding sequence is ambiguous. However, the first 20 amino acids shown are very homologous to the hydrophobic leader sequences previously reported for receptor β -chains¹⁹⁻²¹, indicating that only a few bases of coding sequence are missing. Attempts to sequence this α -chain protein have indicated a blocked N-terminus, possibly the result of the presence of a Gln-derived pyrrolidone carboxylic acid (our unpublished observations). Therefore, we suggest that the mature α -chain sequence begins at the first Gln. The rest of the sequence then encodes a 253-amino acid protein of relative molecular mass (M_r) ~30,000 (30K) with five possible sites for N-linked glycosylation (Asn-X-Ser or Asn-X-Thr). This is consistent with the known M_r (47 K) of the fully glycosylated HPB-MLT α -chain present on the cell surface^{18,23}.

When the sequence of the mature α -chain protein was compared with that of T-cell receptor β -chains, immunoglobulin light and heavy chains and another T-cell-derived immunoglobulin-like molecule of unknown function (pHDS4)²⁸ areas homologous to *V* region, *J* segment and *C* region could be identified. For the *V* region homologies were most apparent in areas corresponding to the three framework regions of immunoglobulin *V* regions defined by Cys at positions 22 and 91 and the Trp at position 35 (Fig. 4a). The homology in the area just before the second Cys is particularly striking. As we pointed out previously²³, based on a partial α -chain protein sequence, the region corresponding to the *J* segment was strongly homologous to other *J* segments, particularly to two receptor β -chain *J* segments (Fig. 4b). There is no obvious evidence for a *D* segment between the *V* and *J* segments of this α -chain, although this possibility cannot be ruled out without knowledge of the germ-line sequences of the *V* and *J* segments.

The *C*-region of the α -chain contains a domain of 51 amino acids defined by Cys at position 135 and 185. This domain is considerably smaller than the corresponding domain in immunoglobulin chains (61-67 amino acids), T-cell β -chains (62-66 amino acids) or the pHDS4 protein (56 amino acids). The sequence homology to these other proteins in the Cys areas is shown in Fig. 4c. There is some homology to β -chains, but very weak homology to the other proteins. We postulate that the third Cys in the *C*-region is the site of disulphide linkage to the β -chain, as there is a Cys in a corresponding position in β -chain *C* regions. There is no homology to β -chains in this region (Fig. 4c), but some homology to the corresponding area of immunoglobulin heavy-chain hinge regions involved in disulphide linkage to light chains is apparent.

The C-terminal end of the protein contains a hydrophobic stretch of 20 amino acids followed by a final Arg-Trp-Ser-Ser. We postulate that this stretch represents a transmembrane and cytoplasmic segment respectively. The presence of two charged basic amino acids in a transmembrane segment is unusual. A charged basic amino acid is also found in the transmembrane segment of β -chains¹⁹⁻²¹. As the receptor is associated in the membrane with the proteins which make up the T3 complex^{11,29}, we speculate that these basic amino acids may be involved in this interaction.

Our results suggest that the sequence and genetic organization of this T-cell receptor α -chain identify it as a member of the rearranging immunoglobulin-like family of genes. This family now has six identified members: α , β and the pHDS4 protein in T cells and heavy chains; λ and κ in B cells. The dissimilarities among the T-cell- and B-cell-derived sequences indicate their very early evolutionary divergence. This collection of cloned genes may elucidate some fundamental questions on T-cell recognition of antigen, such as the structural basis of MHC restriction of antigen recognition, the selection of the T-cell repertoire in the thymus and the nature of self-tolerance.

Since the submission of this manuscript two additional reports of murine T-cell-derived immunoglobulin-like cDNAs have appeared^{30,31}. The *C*-region of the protein encoded in these cDNAs is very similar to that of the human α -chain reported here. The human and murine sequences are identical at 93 of

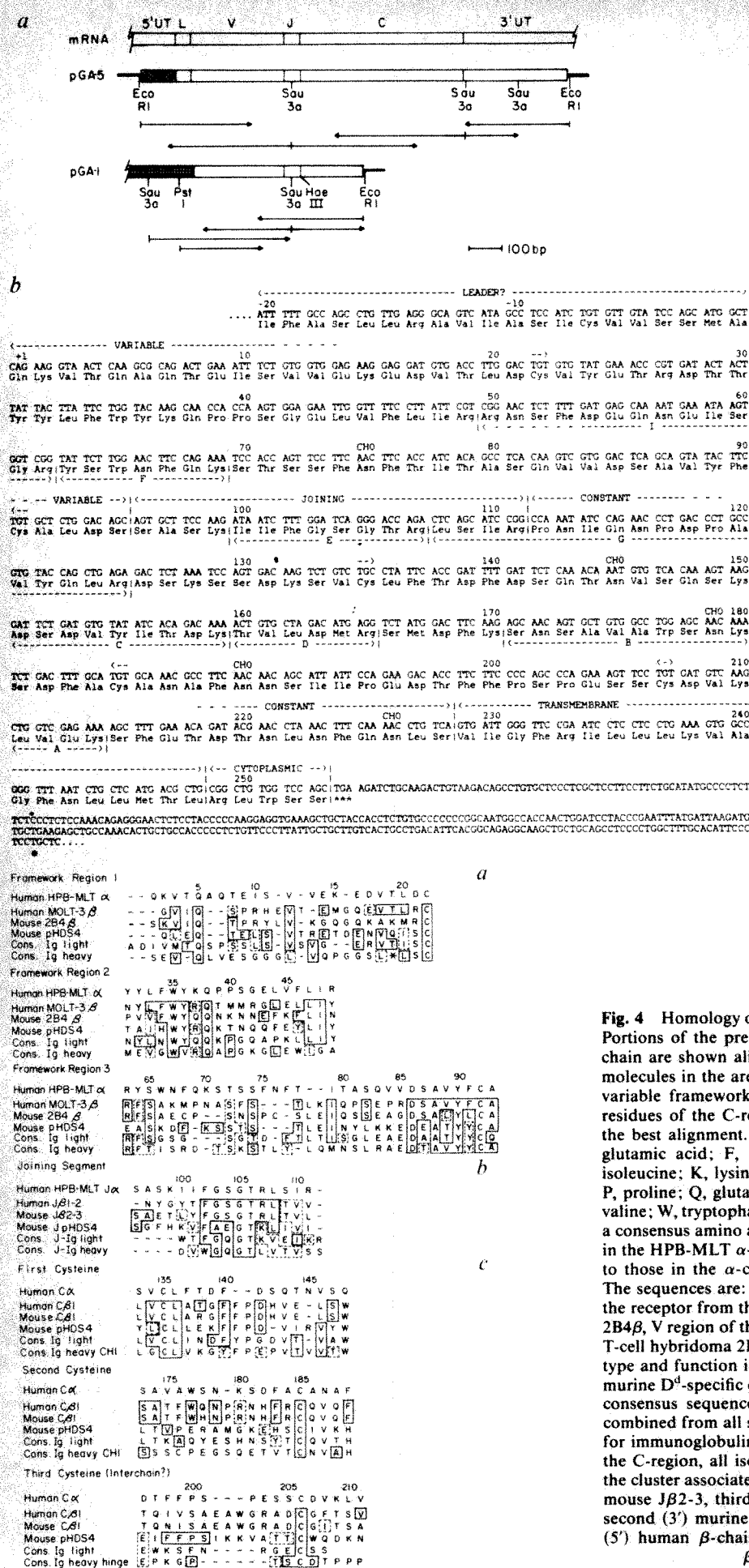


Fig. 3 Nucleotide sequence and predicted amino acid translation of α -chain cDNA. **a**, The inserts of pGA-1 and pGA-5 were isolated. These inserts and fragments generated with several restriction enzymes were subcloned into the sequencing vectors M13mp10 and M13mp11³⁵ and sequenced using the chain termination method of Sanger *et al.*³⁶ from the sites indicated. Thick bar, insert in each plasmid; open areas, derived from α -chain mRNA with identical sequences. The insert in pGA-1 was 1.45 kb. The 5' 950 bp (hatched area) were deduced to be non- α in origin based on the failure of this fragment to detect the T-cell-specific 1.5-kb mRNA (Fig. 2a) and to contain an open reading frame with an interpretable translation. Presumably the pGA-1 insert contains two different cDNAs which were ligated during cloning. Likewise, the first 100 bp of the 1.3-kb insert of pGA-5 (hatched area) could not be ascribed to an α -chain mRNA, as an initiation AUG codon was absent and there was a termination TAA codon just prior to the Ile ATT codon shown. Again a ligation between two DNAs during cloning is a possible explanation for this clone. **b**, The translation of the α -encoding cDNA sequence. Numbering of the protein is based on the assumption that the mature protein starts at the first Gln. Sequenced tryptic peptides of this α -chain are bracketed. The Cys residues presumably involved in disulphide loops ($\rightarrow$, $\leftarrow$) and in linkage to the β -chain ($\leftrightarrow$) are indicated. Asn residues potentially involved in N-linked glycosylation are indicated (CHO). Areas of the protein predicted to be leader, V, J, C, transmembrane, and cytoplasmic segments are indicated, based on homologies with immunoglobulin light and heavy chains, T-cell receptor β -chains, and the pHDS4 protein (see text and Fig. 4).

Fig. 4 Homology of HPB-MLT α -chain to other related proteins. Portions of the predicted protein sequence of the HPB-MLT α -chain are shown aligned with that of other immunoglobulin-like molecules in the areas corresponding to **a**, three immunoglobulin variable framework regions; **b**, the J segment; **c**, the three Cys residues of the C-region. Gaps have been introduced to achieve the best alignment. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histamine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; *, variability too great to assign a consensus amino acid. Solid lines, amino acids identical to those in the HPB-MLT α -chain; dashed lines, amino acids not identical to those in the α -chain, but judged to be conservative changes. The sequences are: human MOLT-3 β , V region of the β -chain of the receptor from the human leukaemia MOLT-3 (ref. 21); mouse 2B4 β , V region of the β -chain of the murine cytochrome c-specific T-cell hybridoma 2B4 (ref. 25); mouse pHDS4, chain of unknown type and function identified from cDNA clones derived from the murine D^d-specific cytotoxic T-cell line C2 (ref. 37); cons. Ig light, consensus sequence for immunoglobulin light chains (κ and λ combined from all species)³⁵; cons. Ig heavy, consensus sequence for immunoglobulin heavy chains (all species and, in the case of the C-region, all isotypes)³⁸; human J β 1-2, second J segment of the cluster associated with the first (5') human β -chain C region²⁷; mouse J β 2-3, third J segment of the cluster associated with the second (3') murine β -chain constant region²⁵; human C β 1, first (5') human β -chain C region²¹; murine C β 1, first (5') murine β -chain constant region^{19,20}.

142 amino acids in the C-region. This high degree of similarity makes it likely that the murine cDNAs do in fact encode receptor α -chains.

We thank J. Cook, J. Stobo, B. Richardson, A. Hayward and D. Geyer for cell lines, W. Born for help in the DNA sequencing, J. White for technical assistance and Edna Squillante and Kelly Bakke for secretarial assistance. This work was supported by

USPHS grants AI-17134, HD-17717, AI-19775, AI-19760 and ACS research grant IM-49. This work was done during the tenure of an ACS Faculty Research Award (number 218) by J.W.K. and whilst J.Y. was on leave from the Servei d'Immunologia, Hospital Clinic, Barcelona, Spain, partially supported by a fellowship from CIRIT, Generalitat de Catalunya, Spain.

Received 12 November; accepted 20 November 1984.

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T cell and monocyte-derived burst-promoting activity directly act on erythroid progenitor cells

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While the terminal stages of erythroid differentiation are regulated by the hormone erythropoietin, the early stages of proliferation and differentiation of immature erythroid progenitor cells also depend on cellular factors functionally defined as burst-promoting activity (BPA)¹⁻⁴. Thus, *in vitro* there is suboptimal development of primitive erythroid progenitor cells (burst-forming units—erythroid, BFU-E) into colonies unless a source of BPA is added. It has been demonstrated that T cells⁵⁻⁸ and monocytes⁸⁻¹⁰ produce BPA. Monocytes may represent the main source of BPA and the major role of T cells may be to augment BPA production by monocytes¹⁰. Irradiated bone marrow cells, which contain T cells, monocytes and other BPA-producing cells, also promote BFU-E colony formation¹¹. As these studies used crude BFU-E populations as target cells, it was not possible to define which of the accessory cell products act directly on the progenitor cell. Here we have used a panel of monoclonal antibodies to purify BFU-E from peripheral blood. We demonstrate that BPA produced by both a monocyte and a T-cell line acts directly on the erythroid progenitor cell and can support colony formation by single BFU-E.

Conditioned media containing BPA were obtained from three sources: Mo cells (a T-cell line)¹², giant cell tumour cells (GCTC, a monocytoïd cell line)¹³ and a single batch of human bone marrow conditioned medium. The three conditioned media all have significant levels of BPA, the highest apparent levels being found in the bone marrow conditioned medium. In three experiments the increase in numbers of BFU-E from adherent cell-depleted (Ad⁻), T cell-depleted, peripheral blood mononuclear cells, produced by 10% Mo cell conditioned medium, GCTC and bone marrow conditioned medium was 113, 70 and 133%, respectively. In separate experiments, all three conditioned

media exhibited maximal burst-promoting activity at a concentration of 10% (v/v).

These media were tested against highly purified BFU-E obtained from peripheral blood. Peripheral blood mononuclear cells were cultured from a panel of 15 normal individuals and the three with the highest incidence of BFU-E in the blood were used for later purification procedures. Adherence cell-depleted, T cell-depleted mononuclear cells (Ad⁻E⁻) were prepared from these individuals and cells expressing differentiation antigens characteristic of mature T cells, B cells, monocytes, granulocytes and natural killer (NK) cells were removed by immune-rosetting (Table 1)^{14,15}. The frequency of BFU-E-derived colonies in cultures of the different cell fractions was determined using irradiated bone marrow cells as a source of BPA. In four experi-

Table 1 Monoclonal antibodies used in the purification of BFU-E

Antibody	Specificity
UCHT1*	Pan T
Leu 5†	Pan T
Leu 2a†	'Suppressor' T cells
Leu 3a†	'Helper' T cells
BA1†	B cells and granulocytes
B1‡	B cells
Leu M1†	Monocytes, granulocytes and NK cells
Leu M2†	Some monocytes
Leu M3†	Some monocytes
TG1§	Granulocytes, some monocytes
Leu 7†	NK cells, some T cells

Peripheral blood mononuclear cells from individuals with more than 60 BFU-E per 10⁵ mononuclear cells, were subjected to a rapid (30 min) adherent cell depletion, followed by removal of cells rosetting with 2-aminoethylisothiuronium bromide (AET)-treated sheep red blood cells. The remaining Ad⁻E⁻ cells were incubated with the panel of monoclonal antibodies shown for 30 min at 4 °C. These antibodies react with T cells, B cells, monocytes and NK cells but not with erythroid progenitor cells. The cells were then washed three times and incubated with sheep erythrocytes to which had been coupled affinity-purified rabbit anti-mouse immunoglobulins (Zymed)¹⁴. Cells binding monoclonal antibodies (rosette-forming cells) were then removed on a Ficoll-Hypaque gradient (specific gravity 1.077). In four experiments the mean number of cells forming immune rosettes was 91%. Antibody sources were as follows: * P. C. L. Beverley¹⁷; † Becton Dickinson; ‡ L. Nadler¹⁹ (used only in expts 3 and 4); § P.C.L. Beverley²⁰.

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ments, the incidence of BFU-E-derived colonies in cultures of the Ad⁻E⁻MAB⁻ fraction (MAB⁻, unreactive with monoclonal antibody) was $11.8 \pm 5.1\%$ (Table 2). In experiments 3 and 4 of Table 2, cells expressing HLA-DR antigens were isolated from the Ad⁻E⁻MAB⁻ fraction; the incidence of BFU-E-derived colonies was increased further by more than twofold. In experiments 3 and 4, the DR⁺ progenitor-enriched cell fractions were diluted to 10 cells per ml and plated in 0.1-ml aliquots into microtitre plates; irradiated bone marrow cells were again added. In experiment 3, 16 of 80 wells developed a single BFU-E colony, as did 17 of 96 in experiment 4. The effects of the three conditioned media on these highly purified BFU-E are shown in Fig. 1. There was potent burst-promoting activity in the Mo cell conditioned medium and the GCTC, but only minimal activity in the bone marrow conditioned medium. In the absence of an added source of BPA, the colonies were small (several hundred cells) with poor haemoglobinization. The Mo cell conditioned medium and the GCTC increased both the size of BFU-E (~10,000 cells per BFU-E colony) and their haemoglobin content, as well as the number of colonies formed. In concurrent experiments using crude Ad⁻E⁻ peripheral blood leukocytes as targets, the bone marrow conditioned medium had BPA activity comparable with that seen in the Mo cell conditioned medium and the GCTC.

To confirm that the Mo cell conditioned medium and GCTC acted directly on the progenitor cells, purified progenitor cells from experiment 4 were also cultured in microwells (1 cell per well) by the method of limiting dilution. Table 3 shows that Mo cell conditioned medium and GCTC were indeed able to support the development into colonies of BFU-E grown at these concentrations, approaching one nucleated cell per well.

These results clearly show that the products of human T-cell and monocyte cell lines exert their burst-promoting activities directly on the erythroid progenitor cells, but do not indicate whether these activities are derived from the same molecular species. It is a reasonable assumption that fresh T cells and monocytes also produce directly acting BPA. Established cell lines were used in this study to overcome the potential difficulties with minor cell contaminants in freshly prepared cell preparations.

It is of considerable interest that the batch of bone marrow conditioned medium used in this study, which had high levels of apparent burst-promoting activity, failed to stimulate colony growth by purified BFU-E and therefore must act indirectly to enhance progenitor cell growth in the impure BFU-E populations. Clearly, other batches of bone marrow conditioned

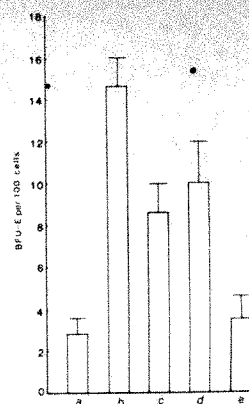


Fig. 1 Effect of irradiated bone marrow cells and conditioned media on the growth of BFU-E from highly purified cell fractions. a, No addition; b, irradiated bone marrow cells; c, Mo cell conditioned medium; d, GCTC; e, bone marrow conditioned medium. Purified progenitors were cultured in methylcellulose in the presence of 2 units of erythropoietin ml^{-1} as described in Table 2. Irradiated bone marrow cells were added at $2.5 \times 10^4 \text{ ml}^{-1}$. Mo cell conditioned medium was given by Dr D. Golde; GCTC was obtained from Gibco; the bone marrow conditioned medium was made by incubating normal low-density bone marrow cells at $2 \times 10^6 \text{ cells ml}^{-1}$ in IMDM plus 10% FCS for 6 days. Conditioned media were all added to a final concentration of 10% (v/v). Conditioned medium from another T-cell line (Jurkatt) was also tested in two experiments and showed no burst-promoting activity against these purified progenitor cells.

medium may have predominantly directly acting BPA, but great caution must be exercised when preparing burst-enhancing factors from such a heterogeneous cell source. The Mo cell conditioned medium and GCTC may also contain indirectly acting factors but this was not determined in our study. It is difficult to ascertain the significance of indirectly acting factors which enhance BPA production, but it has been shown that the serum from aplastic anaemia patients increases leukocyte BPA production compared with normal serum¹⁰. The cellular origin of indirectly acting burst-enhancing factors such as this is unknown. T cells are certainly one possible source¹⁰. We have shown previously that bone marrow contains three populations of cells which enhance BFU-E growth: T cells, monocytes and a third population of lymphoid cells which express Fc receptors for IgG¹¹. It is unknown whether this third accessory cell popula-

Table 2 Purification of BFU-E from peripheral blood

Cell fraction	No. of BFU-E per 10^5 cells plated			
	Expt 1	Expt 2	Expt 3	Expt 4
F/H	133.5	75	77	76
Ad ⁻ E ⁻	777	322	282	180
Ad ⁻ E ⁻ MAB ⁻	17,228	14,200	10,500	5,286
Ad ⁻ E ⁻ MAB ⁻ DR ⁺	—	—	23,200	16,600

The number of BFU-E per 10^5 cells is shown for each cell fraction. F/H, Mononuclear cells prepared by Ficoll-Hypaque density separation from whole blood. BFU-E colonies were grown in 0.9% methylcellulose in Iscove's modified Dulbecco's medium (IMDM; Gibco) with 30% fetal calf serum (FCS), 0.9% deionized bovine serum albumin (BSA), 10^{-4} M β -mercaptoethanol and 2 U ml^{-1} crude erythropoietin (Connaught Step III)²¹. Irradiated bone marrow cells (3×10^5) served as a potent source of BPA. Irradiated bone marrow cells cultured alone gave rise to no BFU-E colonies. In experiments 3 and 4, the antibody-negative cells (MAB⁻) (<3% positive) were subsequently reacted with a monoclonal antibody^{1,2} to HLA-DR²² and immune rosettes formed. The cells were then fractionated on isotonic Percol (pH 7.4, specific gravity 1.085), and the DR-positive (DR⁺) cells in the pellet were freed of red cells by ammonium chloride lysis. Cells from the final two fractions were plated at $5 \times 10^2 \text{ ml}^{-1}$. The yield of BFU-E in these experiments varied between 21 and 45%. Some of these losses (mean 18%) could be readily accounted for by cells removed for cell counting.

Table 3 BFU-E growth from progenitor-enriched fraction

Additive	No. of wells plated	No. of BFU-E
None	94	0
SRBC	95	0
SRBC + Mo cell conditioned medium	95	5
SRBC + GCTC	95	7
SRBC + irradiated bone marrow	92	17

The growth of BFU-E colonies from highly purified progenitor cell populations, plated at a concentration approaching 1 cell per well, depends on the addition of Mo cell conditioned medium, GCTC or irradiated bone marrow cells. Note that the BFU-E growth in microwells was slower than that in Petri dishes and the BFU-E attained a smaller final size. The culture medium was identical to that described in Table 2 except that 2×10^5 washed sheep red blood cells (SRBC) were added to each well as previous experiments had shown that, at very low cell numbers, the growth of BFU-E in microwells was very poor without some other source of 'filler' cells. Red cells were chosen as these are inert and contain no BPA activity: for example, in experiment 4, the number of BFU-E growing from the progenitor-enriched fraction plated at $5 \times 10^2 \text{ ml}^{-1}$ in 1-ml Petri dishes was 1,200 per 10^5 without any added cells or medium, 1,300 per 10^5 with sheep red blood cells added and 16,600 per 10^5 with irradiated bone marrow cells.

tion produces directly or indirectly acting factors—further studies are required to address this issue.

The study reported here shows that BPA produced by a T-cell and a monocyte cell line acts directly on committed erythroid progenitors whereas other bone marrow-derived factors may act indirectly; it illustrates the complexity of cell interactions involved in the regulation of erythropoiesis, and underlines the importance of using pure populations of progenitor

cells to elucidate these events. The purity of BFU-E obtained in this study is considerably greater than that described previously^{17,18} and this should facilitate further understanding of the regulation and differentiation of erythroid progenitor cells.

This work was supported in part by grants from the NIH and the Dyson Foundation. D.C.L. was a Wellcome Research Fellow and was in receipt of a MRC Travelling Fellowship.

Received 21 May; accepted 4 October 1984.

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Variant (6;15) translocation in a murine plasmacytoma occurs near an immunoglobulin κ gene but far from the *myc* oncogene

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Chromosome translocations in B-lymphoid tumours are providing intriguing insights and puzzles regarding the role of immunoglobulin genes in the activation of the *myc* oncogene (reviewed in refs 1, 2). The 15;12 translocations found in most murine plasmacytomas and the analogous 8;14 translocation in human Burkitt's lymphomas involve scissions of murine chromosome 15 (human chromosome 8) near the 5' end of the *c-myc* gene and subsequent fusion near an immunoglobulin heavy-chain gene. The less well characterized 'variant' translocations found in about 15% of such tumours also involve the *myc*-bearing chromosome band, but exchange occurs with a chromosome bearing an immunoglobulin light-chain locus—in mice, the κ -chain locus bearing chromosome 6 (refs 3-5) and, in man, chromosome 2 (or 22), at the same band at which the κ (or λ) locus lies (reviewed in ref. 1). The Burkitt variant translocations involve scissions 3' of *c-myc*⁶⁻¹⁰; one 8;22 translocation placed the *C λ* locus just 3' of *c-myc*¹⁰, but usually the chromosome 8 breakpoint is a greater, but unknown, distance away from *c-myc*, more than 20 kilobases (kb) in one 8;22 translocation involving the *C κ* gene⁹. Little is known about the murine 6;15 translocations, although a *C κ* gene cloned from one plasmacytoma (PC7183) is linked, via chromosome 12 sequences, to an unidentified region of chromosome 15 (ref. 11). We describe here the chromosome fusion region from plasmacytoma ABPC4, which displays the typical reciprocal 6;15 translocations⁴. We find that the chromosome 6 breakpoint is near *C κ* but, unlike those in the heavy-chain locus, not at a position where immunoglobulin genes normally recombine. Moreover, the chromosome 15 sequences involved in the ABPC4 translocation are not derived from the vicinity of *c-myc*.

Because the chromosome 15 breakpoint in a 15;6 translocation is cytogenetically indistinguishable from that in a 15;12 translocation³⁻⁵, we initially expected to find a rearranged *c-myc* gene in ABPC4. However, Southern blot hybridization revealed no alteration within the 20.5-kilobase (kb) *EcoRI* fragment bearing *c-myc* in ABPC4, nor in several other lines with a 15;6 translocation (refs 5, 12 and our unpublished results). Pursuing the hypothesis that the 15;6 translocation generates breaks near *C κ* , we cloned both rearranged *C κ* alleles from this κ -expressing tumour. The rearrangement on one allele proved to be within

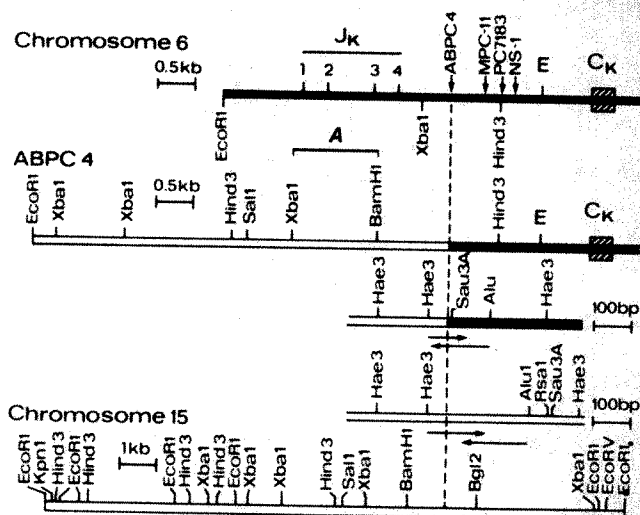


Fig. 1 Restriction endonuclease maps of the germ-line J_{κ} - C_{κ} locus, the ABPC4 C_{κ} gene linked to a chromosome 15 region and the corresponding unrearranged chromosome 15 region. The C_{κ} locus is depicted by a filled bar and chromosome 15 sequences by an open bar. Vertical arrows in the J_{κ} - C_{κ} intron mark points of recombination in ABPC4 and in three other plasmacytomas (see text). E denotes the κ enhancer and A marks the region used as a probe. On the expanded maps, arrows denote the direction and extent of sequencing (see Fig. 3). The hatched box indicates the C_{κ} exon.

Methods: ABPC4 was induced in a BALB/c mouse by pristane and infection with Abelson murine leukaemia virus, which accelerates plasmacytoma formation⁵. Southern blots of ABPC4 DNA with a C_{κ} probe revealed two rearranged *EcoRI* fragments (14.5 and 16.9 kb). Fragments of those sizes, enriched on glycerol gradients, were cloned in phage vector Charon 4A. The 14.5-kb fragment (not shown) presumably bears the active $V_{\kappa}J_{\kappa}C_{\kappa}$ gene in this κ -expressing tumour, as its rearrangement proved to be within the J_{κ} region, in contrast to the intron rearrangement in the 16.9-kb fragment, the 5' portion of which is shown above. When the DNA rearranged to the J_{κ} - C_{κ} intron proved to originate from chromosome 15 (see text), probe A from the chromosome 15 portion was used to isolate the corresponding region of unrearranged chromosome 15 DNA from a library of BALB/c embryonic DNA fragments made by partial *EcoRI* digestion.

the κ -chain joining (J_{κ}) locus, presumably reflecting conventional V_{κ} - J_{κ} recombination. On the other allele, however, rearrangement occurred within the J_{κ} - C_{κ} intron (Fig. 1). The incoming DNA was shown to be derived from chromosome 15 by hybridizing probe A in Fig. 1 to DNA from a panel of mouse-hamster hybrid lines¹³⁻¹⁵ used previously, in collaboration with Dr Uta Francke, to assign *c-myc* to chromosome 15 (ref. 15). The probe hybridized to DNA of the same lines as a

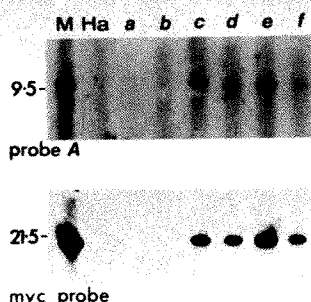


Fig. 2 Blot hybridization data from mouse-hamster cell lines showing that DNA fused to the C_{κ} locus in ABPC4 is derived from chromosome 15. The Southern blots show hybridization with a probe derived from the incoming DNA (A in Fig. 1) and a *c-myc* probe to DNA from BALB/c mouse embryos (M), hamster liver (Ha) and six mouse-hamster cell lines selected to be informative for chromosome 15¹³⁻¹⁵. The sizes of the hybridizing mouse fragments are in kilobases. The cell lines, the full karyotype of which was determined by Francke and colleagues^{13,14} and provided in Table 1 of ref. 15, were as follows: a, I-18A HAT; b, I-18A-2a-8AG; c, EBS 58; d, EBS 11; e, EBS 4; and f, I-13A-1a-8AG. Mouse chromosome 6 is present in good yield in the DNA in lanes a and f, low in b and c and absent in lanes d and e (ref. 15); hence, the results in lanes a, d and e and probably c are inconsistent with derivation of probe A sequences from chromosome 6.

c-myc probe (Fig. 2). The complement of mouse chromosomes in these lines, specified in Table 1 of ref. 15, eliminates derivation of probe A sequences from any mouse chromosome except 15. In particular, results with at least three lines are inconsistent with its being chromosome 6 (see legend to Fig. 2). We conclude that the ABPC4 clone in Fig. 1 bears the 15; 6 fusion region.

The κ locus has previously been assigned to chromosome 6 (ref. 16), but has been localized only by genetic linkage data¹⁷. As cytogenetic studies³⁻⁵ place the chromosome 6 breakpoint at band C2, our evidence that C_{κ} lies at the breakpoint maps the κ locus to that band, placing κ about 0.4 of the distance from the centromeric end to the distal end of the chromosome, in good agreement with the linkage map¹⁷.

To analyse the nature of the recombining sequences, we determined sequences across the 15; 6 fusion point in ABPC4 and across a corresponding region of unrearranged chromosome 15 DNA cloned from embryos (Fig. 1, bottom). Figure 3 compares these sequences with that in the J_{κ} - C_{κ} intron¹⁸. Recombination occurred 1.85 kb 5' to C_{κ} , that is, 665 base pairs (bp) 3' to the nearest J_{κ} gene. Like the 15; 12 translocation¹⁹⁻²¹, this exchange clearly represents nonhomologous recombination, as the chromosome 15 sequence is not related to the J_{κ} - C_{κ} sequence, except for an AT at the recombination point in both. No repeated sequence elements seem to be involved, as the region around the chromosome 15 (and 6) breakpoints behave as unique sequences in hybridization experiments (Fig. 2 and other blots not shown). Significantly, the heptamer and nanomer 'signal sequences' required for V_{κ} - J_{κ} fusion (reviewed in refs 22, 23) do not occur in the chromosome 15 region sequenced, nor in this portion of the J_{κ} - C_{κ} intron—the heptamer at which a V_{κ} gene aberrantly recombined in MPC-11 (ref. 24) lies 440 bp 3' to the ABPC4 breakpoint (Fig. 1, top). Because V_{κ} - J_{κ} recombination always seems to occur within a few base pairs of the heptamer signal, it seems unlikely that V - J joining enzymes catalysed this 6; 15 translocation or the two best-characterized

examples of Burkitt variant translocations, which involve breakpoints 5' to the J_{κ} (ref. 10) and J_{κ} (ref. 9) regions. Thus, whereas the immunoglobulin switch recombination machinery may well participate in the 15; 12 translocation, particularly in the chromosome 12 scissions²¹, there is no indication that immunoglobulin rearrangement enzymes are involved in the variant translocations.

The ABPC4 translocation differs in several respects from the bizarre product involving sequences from chromosomes 15, 12 and 6 found in plasmacytoma PC7183 (ref. 11). While both involve a chromosome 6 breakpoint within the J_{κ} - C_{κ} intron, that in PC7183 is several hundred base pairs closer to C_{κ} (Fig. 1, top). More importantly, the cloned chromosome 15 region involved in the PC7183 translocation¹¹ (kindly provided by Drs M. Weigert and M. Shapiro) is not related to the 17-kb segment of chromosome 15 described here (Fig. 1, bottom), as their restriction maps differ and the PC7183 probe did not hybridize to the clones in Fig. 1. Thus, variant translocations can involve different chromosome 15 breakpoints. The most dramatic difference between the ABPC4 and PC7183 translocations, however, is that whereas sequences from chromosomes 15 and 6 directly abut in ABPC4 (Fig. 3), in PC7183 they are separated by 1 kb of the immunoglobulin heavy-chain locus (S_{μ} region)¹¹ from chromosome 12. Thus, PC7183 must have undergone multiple recombination events, whereas ABPC4 represents a simple exchange. From the cytogenetics of tumours like ABPC4³⁻⁵, one would expect a reciprocal translocation product in ABPC4, and indeed we have detected such a product, which will be described elsewhere.

The region 5' to the C_{κ} gene seems to be prone to aberrant recombination events. An atypical translocation linking C_{κ} to chromosome 10 has been described in plasmacytoma NS-1 (ref. 25) and its breakpoint lies only 180 bp from that in PC7183 (Fig. 1, top). In another plasmacytoma, an intercisternal A-particle element has become inserted ~0.1 kb 5' to the ABPC4 breakpoint²⁶, while two others have deletions across C_{κ} which end within the J_{κ} - C_{κ} intron²⁷. These findings suggest that the J_{κ} - C_{κ} region is a recombinational 'hot spot' in B lymphocytes. This propensity might be related to its involvement in V_{κ} - J_{κ} assembly, to the constitutive C_{κ} expression in B cells²⁸ and/or to the nuclease hypersensitivity²⁹⁻³² associated with the C_{κ} enhancer region (see below).

The *c-myc* gene is transcribed in ABPC4 at levels comparable to that in other plasmacytomas (refs 5, 12 and our unpublished results). As only the *c-myc* allele involved in a translocation is active in most lymphoid tumours^{6,7,20,34,35}, it seems very likely that the 15; 6 translocation has induced *myc* expression in ABPC4. Hence, it is intriguing that the ABPC4 breakpoint is not near the *myc* gene. Comparison of the 17-kb segment of chromosome 15 defined in Fig. 1 (bottom) with clones spanning 20.5 kb of the *c-myc* locus¹⁹ places the ABPC4 breakpoint more than 20 kb from the *c-myc* promoters, and other clones (our unpublished results) place it more than 50 kb away, depending on whether it lies 5' or 3' of *c-myc*. In striking contrast, the vast majority of 15; 12 translocations break the *c-myc* transcriptional unit¹⁹. This marked difference may mean that the 15; 12 and 15; 6 translocations activate *myc* by different mechanisms.

Translocation to C_{κ} would be favoured in a B lymphocyte if the C_{κ} region induces expression of an incoming gene, as it does for the V_{κ} gene³⁶. Induction of V_{κ} transcription requires proximity to the enhancer (E in Fig. 1) located 0.6 kb 5' to C_{κ} .

Fig. 3 Sequences involved in the 6; 15 translocation in ABPC4. Chromosome 6 sequences are given in italics and chromosome 15 in standard typeface. Due to the AT on both chromosomes at the recombination point, cross-over could have occurred between any of three residues, as indicated. The chromosome 15 G residue marked with an asterisk has been converted to an A residue in ABPC4, presumably as a consequence of the translocation, but no other alterations were found from the germ-line chromosome 15 or chromosome 6 sequence.

Methods: Detailed restriction maps near the junction (Fig. 1) localized the recombination points, and appropriate fragments were inserted into the single-stranded phage vector M13mp10 or M13mp11 (ref. 39). Sequences were determined by the dideoxynucleotide method⁴⁰.

(refs 37, 38). A direct effect of the C_{κ} enhancer on *c-myc* transcription in ABPC4 appears unlikely, however, because of the large distance involved. Nevertheless, the C_{κ} locus might generate long-range effects along the translocated chromosome (in *cis*) by perturbation of the chromatin suprastructure. Alternatively, the variant translocation might induce *myc* expression in *trans* through an effect on a gene regulating *myc* expression.

We thank Dr M. Potter and Litton Bionetics for the ABPC4 tumour, Drs M. Weigert and M. Shapiro for the PC7183 clone, S. Gerondakis for the embryonic library, and Leonie Gibson and Jan Mitchell for technical assistance. This work was supported by the National Health and Medical Research Council (Canberra), the US National Cancer Institute (CA12421), the American Heart Association, and the Drakensberg Trust.

Received 31 August; accepted 11 October 1984.

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Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells

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The effects of steroid hormones are mediated by intracellular hormone-specific receptor proteins; the interaction between the hormone and its receptor increases the affinity of the receptor for nuclear binding sites, thereby modulating the expression of specific genes¹. The glucocorticoid receptor is a soluble protein of relative molecular mass (M_r) 94,000 (94K), present at a low relative abundance ($\leq 0.01\%$); it has been purified to near-homogeneity^{2,3}, and specific antisera and monoclonal antibodies have been produced⁴⁻⁶. Purified glucocorticoid receptor binds *in vitro* with high affinity to defined regions of DNA near regulated promoters⁷⁻¹⁰, and sequences essential for these interactions are functional *in vivo* as hormone-dependent transcriptional enhancer elements^{11,12}. We have now cloned complementary DNA (cDNA) for the rat liver glucocorticoid receptor and we describe here a 2.6-kilobase (kb) receptor cDNA isolated following polysome immuno-enrichment of receptor messenger RNA with glucocorticoid receptor-specific antibodies. The receptor appears to be encoded by a single-copy gene which specifies a ~6-kb transcript in rat and mouse cells; this mRNA is altered quantitatively and qualitatively in several mutant cell lines with specific defects in receptor function.

Polysomes and RNA were isolated from J2.17 cells, a mammary tumour virus (MTV) infected derivative of the rat hepatoma HTC line¹³. Figure 1 compares *in vitro* translation products either from total J2.17 poly(A)⁺ RNA (lane 2) or from a receptor mRNA-enriched preparation (lane 3); in general, the ³⁵S-methionine-labelled proteins obtained from the two reactions are similar, although several species are more strongly labelled in lane 3. Three proteins specified by the enriched RNA were immunoprecipitated by receptor-specific monoclonal antibody (lanes 4, 5); two of these (the 94K and 79K species) were

readily observed among the total proteins synthesized from the enriched mRNA, while the third, a 91K protein, was detected only after precipitation. We were encouraged by this finding, because purified preparations of the 94K; rat liver glucocorticoid receptor often contain two discrete proteolytic degradation products which migrate as minor 91K protein and a more abundant 79K species³.

We calculated that 2% of the *in vitro* translation product from the immunologically purified RNA was receptor protein, and assumed that receptor mRNA was present at comparably high levels in that RNA preparation. Therefore, we constructed a cDNA library¹⁴, beginning with 0.4 μ g of enriched mRNA; 540 transformed bacterial colonies were screened by differential hybridization with labelled cDNA probes from enriched and unenriched RNA. Six independent clones were identified and shown to contain inserts of between 0.9 and 3.5 kb; all of the inserts cross-hybridize, and restriction mapping suggests that they represent overlapping clones from a single transcription unit (R.M. *et al.*, manuscript in preparation). In this study, we used a plasmid with a 2.6-kb insert (pRM16), shown by DNA sequencing to contain a segment of open reading frame and a putative poly(A)-addition signal (AATAAA) upstream of an 80-nucleotide poly(A) tract (S. Rusconi, unpublished data). Northern hybridizations revealed a ~6-kb transcript homologous to the pRM16 insert (see below) that had been selectively enriched ≥ 200 -fold by polysome immunoadsorption, corresponding closely with predictions based on the relative abundance of receptor translated from total poly(A)⁺ RNA ($\leq 0.01\%$) and from enriched RNA (2%).

Hybrid-selected translation¹⁵ was used to confirm that pRM16 contains sequences complementary to receptor mRNA. Enriched RNA was hybridized with pRM16, and as a control, with a β -tubulin cDNA plasmid, which were immobilized on separate nitrocellulose filters; RNA recovered from each filter was then translated *in vitro*. pRM16-selected mRNA yields translation products indistinguishable from intact receptor and its two apparent degradation products (Fig. 2); a comparison of lanes 1 and 3 of Fig. 2 reveals that the hybrid-selection reaction gave an overall recovery of about 10% of the intact receptor-coding RNA initially added, as expected for this procedure¹⁶.

The labelled pRM16 insert was used to probe Southern blots of genomic DNA from cultured rat cell lines of liver (J2.17) or fibroblast (XC) origin. Figure 3 shows that DNA from these two cell types yielded identical hybridization patterns after digestion with *Bam*HI (lanes 1, 4), *Eco*RI (lanes 2, 5) or *Hind*III (lanes 3, 6). Moreover, only two fragments were observed in

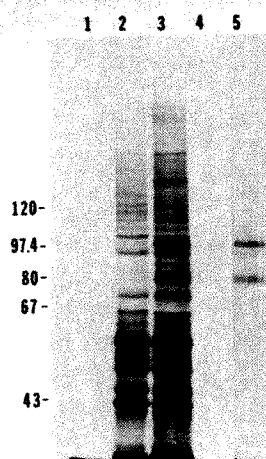


Fig. 1 Immunoprecipitation of ^{35}S -methionine-labelled receptor translated *in vitro*. Total translation products were from reactions programmed with no RNA (lane 1), 1 μg total poly(A) $^{+}$ RNA (lane 2), or $\sim 0.3 \mu\text{g}$ receptor-enriched RNA (lanes 3, 4) preparations. Products in lane 4 were immunoprecipitated with receptor-specific monoclonal antibody; lane 5, sixfold longer exposure of lane 4. No labelled proteins were recovered in a parallel precipitation with control ascites, or after precipitation of total poly(A) $^{+}$ RNA translation products with receptor antibodies (data not shown). Relative molecular mass standards ($\times 10^{-3}$), top to bottom: β -galactosidase, phosphorylase *b*, conalbumin and ovalbumin. Electrophoresis was in a 7% SDS-polyacrylamide gel; lanes 1 and 2 received 5% of the respective reaction products; lane 3, 15%; lane 4, 85%.

Methods: Polysome immunoadsorption was carried out essentially as described elsewhere^{28,29}; 2×10^9 J2.17 cells yielded about 1,000 A_{260} units of polysomes. A 1:1 mixture of a receptor-specific antiserum⁴ and a monoclonal antibody (IgG2a, no. 7; ref. 6) was purified with protein A-Sepharose to remove ribonucleases; 7 mg of the antibodies was incubated for 1–2 h at 4°C with 1,000 A_{260} units of polysomes in 30 ml of polysome buffer (25 mM Tris, pH 7.5, 5 mM MgCl_2 , 0.1% Nonidet P-40, 150 mM NaCl, 1 $\mu\text{g ml}^{-1}$ Trichodermin (Leo) and 0.5 mg ml^{-1} heparin), then passed twice through a 5-ml protein A-Sepharose column. The column was washed overnight with 200 ml polysome buffer followed by RNA elution with 20 ml of 25 mM Tris, pH 7.5, 20 mM EDTA, 0.5 mg ml^{-1} heparin; poly(A) $^{+}$ RNA was subsequently purified by oligo(dT)-cellulose chromatography, then ethanol precipitated in the presence of 10 $\mu\text{g ml}^{-1}$ bovine liver tRNA and 2 M ammonium acetate. About 2 μg of enriched RNA was recovered, as estimated from *in vitro* translation of known amounts of J2.17 total poly(A) $^{+}$ RNA. Reaction conditions for *in vitro* translation were exactly as described by the suppliers (Bethesda Research Laboratories or Promega Biotec) using 100 μCi ^{35}S -methionine (NEN 1,000 Ci mmol^{-1}) in 50 μl final volume. Immunoprecipitations³⁰ were done using monoclonal antibody IgG2a, no. 7 (ref. 6); control precipitations (not shown) used ascites fluid from mice injected with Sp2/0 myeloma cells, the parent line used in the hybridoma fusions⁶.

each digest, consistent with the notion that the receptor is encoded by a single-copy gene.

An extensive series of mouse lymphoma cell mutants have been isolated which fail to respond to glucocorticoids and carry certain distinct receptor defects: (1) receptor-deficient (r^{-}) mutants lack steroid-binding activity; (2) nuclear-transfer deficient (nt^{-}) lines produce a 94K M_r hormone-binding protein that interacts with reduced affinity with both nonspecific^{17,18} and specific¹⁹ DNA sequences; (3) increased nuclear-transfer (nt^i) mutants contain a hormone-binding species 40K M_r that lacks a major antigenic determinant²⁰, associates with non-specific DNA with increased affinity¹⁷ and binds specific DNA sequences with reduced affinity²¹. To determine whether the receptor alterations reflect lesions detectable at the RNA level, receptor transcripts from several such cell lines were compared by blot hybridization. These included two wild-type mouse lymphoma cell lines, WEH17 (ref. 22) and S49.1 (ref. 14); the

Fig. 2 Hybrid-selection of receptor RNA. Enriched RNA from $\sim 5 \times 10^8$ cells was hybridized to filters containing 10 μg of immobilized DNA from either a chicken β -tubulin cDNA plasmid (lane 2) or pRM16 (lane 3); these two lanes show immunoprecipitated *in vitro* translation products of the eluted RNA. The small amount of receptor detected in lane 2 probably reflects background hybridization due to relaxed stringency washes used in this hybrid-selection procedure³¹. Lane 1, immunoprecipitated *in vitro* translation products of enriched RNA ($\sim 5 \times 10^8$ cell equivalents) that was not subjected to hybrid-selection. Markers and gel electrophoresis conditions were as in Fig. 1.

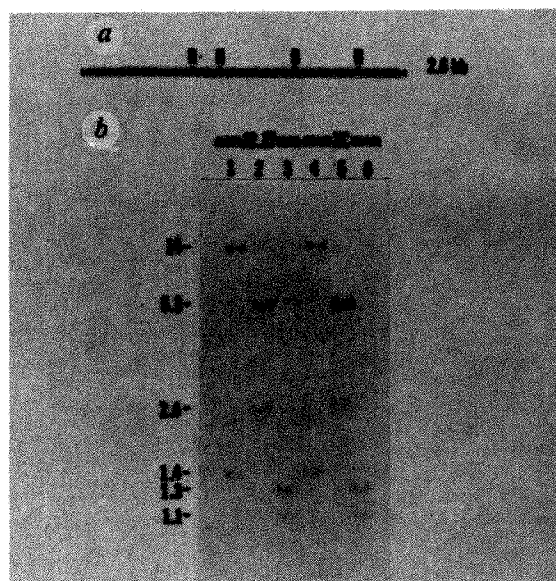
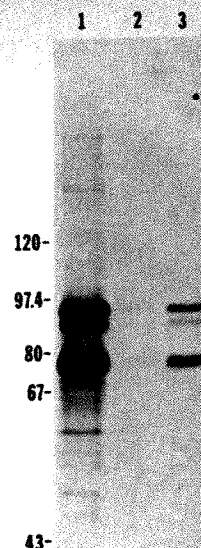


Fig. 3 Complexity of receptor sequences in genomic DNA from liver and fibroblast-derived rat cell lines. *a*, *Bam*HI (B), *Eco*RI (R) and *Hind*III (H) restriction sites within the 2.6-kb pRM16 inserts. Strand-specific cRNA probes used in Northern blot analysis (see Fig. 4) revealed that transcription proceeds from left to right. *b*, Southern blot using 20 μg of HTC (lanes 1–3) or XC (lanes 4–6) DNA was digested with *Bam*HI (lanes 1, 4), *Eco*RI (lanes 2, 5) or *Hind*III (lanes 3, 6), electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and probed with pRM16 using hybridization and wash conditions as previously described²⁷.

mutant lines, all derived from the latter parent, included an nt^{-} mutant, two nt^i derivatives, and one r^{-} line. In addition, a rat HTC cell-derived r^{-} mutant, MSN5.3 (ref. 23), was tested.

Figure 4 shows a Northern blot in which equal amounts of total RNA from these cell lines were hybridized with pRM16 probe; interestingly, the probe detects rat and mouse sequences with apparently equal efficiency. In every case, a transcript of ~ 6 kb is observed, but the relative abundance of this transcript differs over a severalfold range among these cell lines; the same relative quantitative differences were seen in three independent RNA preparations. Thus, WEH17 contains two- to threefold more receptor RNA than S49.1 (lanes 3, 4), while the transcript in the nt^{-} mutant is indistinguishable from that in its parent (lanes 8, 4). In contrast, the nt^i lines accumulate only about half as much of the 6-kb transcript as is found in the parent (lanes 4–6); similarly, the amount of the 6-kb transcript is reduced severalfold in both the mouse lymphoma r^{-} line and the rat

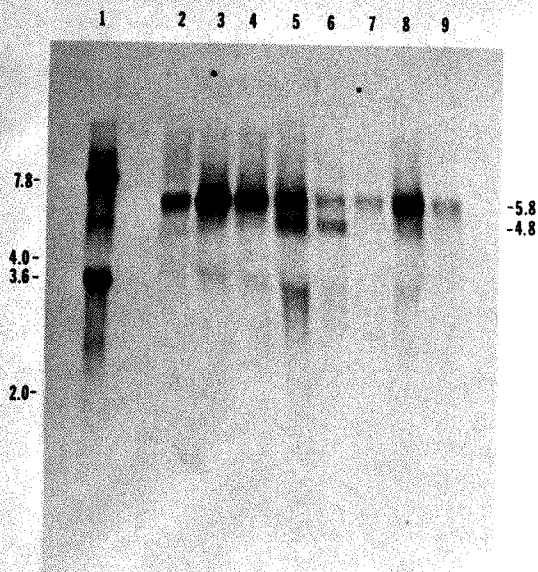


Fig. 4 Northern blot analysis of receptor RNA in wild-type and receptor mutant cell lines. Lanes 1–9 contain 20 µg of total RNA from rat HTC-derived (lanes 1, 2, 9) or mouse lymphoma (lanes 3–8) cell lines. The filter was hybridized with either nick-translated MTV DNA (lane 1) or pRM16 cRNA probe produced by SP6 polymerase³² (lanes 2–9). Cell lines and receptor phenotypes: M1.54, wild type (wt) (lanes 1, 2); WEH17, wt (lane 3); S49.1, wt (lane 4); S49.55R, nt⁺ (lane 5); S49.143R, nt⁺ (lane 6); S49.7R, r⁺ (lane 7); S49.22R, nt⁺ (lane 8); and MSN5.3, r⁺ (lane 9). Equivalent amounts of RNA were loaded in each lane, as assessed by hybridization with β -actin cDNA (not shown). The MTV transcripts (7.8 and 3.6 kb) and rRNAs (4.0 and 2.0 kb) were used as M_r to infer the sizes of the receptor transcripts; electrophoresis was in a 0.9% agarose-formaldehyde gel.

hepatoma r⁺ mutant relative to their respective parent lines (lanes 7 and 4 and lanes 9 and 2). In addition to these quantitative differences, both nt⁺ mutants contain an additional receptor transcript which migrates with an apparent size of ~5 kb and is present in these mutants at about the same relative abundance as the 6-kb transcript (lanes 5, 6). Southern blots of genomic DNAs from these wild-type and mutant cells were indistinguishable when monitored by pRM16 hybridization to *Eco*RI and *Hind*III restriction fragments (data not shown), suggesting that the mutant phenotypes do not reflect gross DNA rearrangements or deletions.

Westphal *et al.*²⁴ have recently analysed receptor protein in a variety of mouse lymphoma cells (including four studied here) using a combination of hormone binding and immunoreactivity measurements. They conclude that WEH17 produces relatively high levels of receptor from both chromosomal homologues, whereas S49, which appears to be functionally hemizygous^{23,25}, produces active receptor from one homologue while the other allele encodes a 94K protein that is immunoreactive but fails to bind steroid. Similarly, r⁺ mutants which lack hormone-binding activity nevertheless produce 20–50% as much 94K immunoreactive material as the wild-type parent. Our qualitative and quantitative characterizations of receptor transcripts correlate directly with those studies. Thus, we suggest that the 6-kb RNA present at reduced levels in r⁺ and nt⁺ mutants may encode the nonfunctional 94K protein, and that the 40K nt⁺ receptor may be encoded by the nt⁺-specific 5-kb transcript. Finally, receptor mRNA in the nt⁺ mutant does not differ detectably from wild type, consistent with the bulk physical properties of this mutant protein¹⁸.

To our knowledge, this is the first reported isolation of sequences encoding a eukaryotic transcriptional regulatory protein whose activity is controlled by a defined physiologically important ligand. Moreover, the specific genomic sites of action have been determined at the nucleotide level for this protein¹², and its binding at these sites shown to regulate the novel process of

transcriptional enhancement^{11,26,27}. The elucidation of receptor gene organization and expression in wild-type and mutant cells will provide valuable information for studies on the mechanisms by which receptor regulates gene expression.

We thank Dave Drubin for advice on polysome immunoenrichment, Gary Firestone for advice on immunoprecipitation, and Anna Wu for contributions to early phases of the project. We also thank Gordon Ringold for MSN5.3 cells, Marc Kirschner for β -actin and β -tubulin cDNAs, Bruce Alberts, Dave Bieber and Ken Zaret for suggestions on the manuscript, and Kathleen Rañeses and Bonnie Maler for preparation of the text and figures, respectively. Research support was from the NSF and NIH (to K.R.Y.) and the Swedish MRC (to J.-Å.G. and to Ö.W.); R.M. was supported by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research, S.O. received funds from the International Cancer Research Data Bank and the International Union Against Cancer for studies at UCSF, and K.R.Y. is a recipient of a Dreyfus Teacher-Scholar Award.

Received 10 September; accepted 26 October 1984.

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Competition for cellular factors that activate metallothionein gene transcription

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Metallothioneins (MTs), small cysteine-rich proteins, bind to and are inducible by heavy metals such as zinc, cadmium and copper. Recent gene-transfer and mutagenesis experiments have elucidated cis-acting DNA sequences involved in this form of regulation^{1,2}, but nothing is known about the trans-acting factors that interact with the control sequences or how such interactions influence the

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rate of transcription. We report here the detection of cellular factors involved in the cadmium induction of the mouse *MT-1* gene by an *in vivo* competition assay. We show that at least one class of these cellular factors acts by a positive regulatory mechanism depending on the same region of the 5' flanking DNA required for maximal transcription.

In *Escherichia coli*, *lac* operator DNA at high copy number results in titration of *lac* repressor and constitutive transcription of the operon³. We have applied the same principle to mammalian cells, namely that if a regulatory factor is present in limited amounts it should be possible to titrate it by introducing sufficient control-sequence DNA into the cells. By determining whether competition leads to a decrease or increase in transcription, one can distinguish between positive regulation by an activator factor and negative regulation by a repressor. Furthermore, by varying the structure of the competitor DNA, it is possible to localize the interacting DNA sequences and to determine whether different genes are regulated by common or distinct factors. A similar approach has been used⁴ to detect cellular factors that interact with molecules containing viral enhancer sequences.

Cultured monkey kidney cells were transfected with a calcium phosphate co-precipitate of three different simian virus 40 (SV40)-based plasmid DNAs: (1) The indicator plasmid contains a mouse *MT-1*-*E. coli* chloramphenicol acetyltransferase (*MT-CAT*) fusion gene in which *MT* regulatory sequences drive the expression of bacterial enzyme coding sequences. This plasmid is used to monitor *MT* gene transcription determined by CAT enzyme measurements⁵. (2) The competitor plasmid, which is used to titrate out the cellular regulatory factors, also contains *MT* regulatory sequences, but these are not fused to the *CAT* gene. In most experiments we used *MT-gal* fusions so that the transcriptional activity of the competitor DNA could be measured in an independent transfection experiment⁶. (3) The carrier plasmid, used to keep the total DNA concentration constant in each precipitation mixture, is identical to the competitor plasmid except that it lacks all of the *MT* regulatory sequences.

Following transfection, cells were incubated with or without cadmium, extracts prepared and CAT enzyme activities measured to determine the transcription levels of the indicator plasmid. Because all of the plasmids contain the origin of SV40 DNA replication (provided by an SV40-guanine phosphoribosyl transferase fusion gene in the indicator plasmid), and because SV40 large T antigen is provided by the competitor and carrier plasmids, these plasmids are capable of replicating in the transfected cells. From Southern blot analyses, we estimated intracellular copy numbers of 7×10^4 molecules of indicator DNA and 1.3×10^6 molecules of competitor plus carrier DNA per cell in these conditions (ref. 6, and data not shown). The plasmids also contain functional SV40 early and late promoters so that each assay includes a high concentration of SV40 control sequences (not regulated by heavy metals) as well as the inducible *MT* regulatory elements. This strategy was adapted to avoid artefacts caused by nonspecific competition for general transcription factors.

The predicted results of this experiment are shown in Fig. 1b. If transcription were negatively controlled by a limiting component, we would expect the competing DNA to titrate out the repressor molecule. Consequently, the basal level of transcription would increase while the induced level would remain constant. In contrast, if the gene were positively controlled by a limiting component, we would expect the competitor DNA to titrate out the activator molecule, resulting in a decrease in transcription in the presence of cadmium. Figure 2 shows the results of an actual competition experiment in which the indicator and competitor plasmids contained the same 2,000 base pairs (bp) of *MT* 5'-flanking sequences. The addition of increasing amounts of competitor DNA led to a sequential decrease in the expression of the indicator plasmid in the presence of cadmium but had little effect on the basal expression levels. This is consistent with titration of positively acting cellular

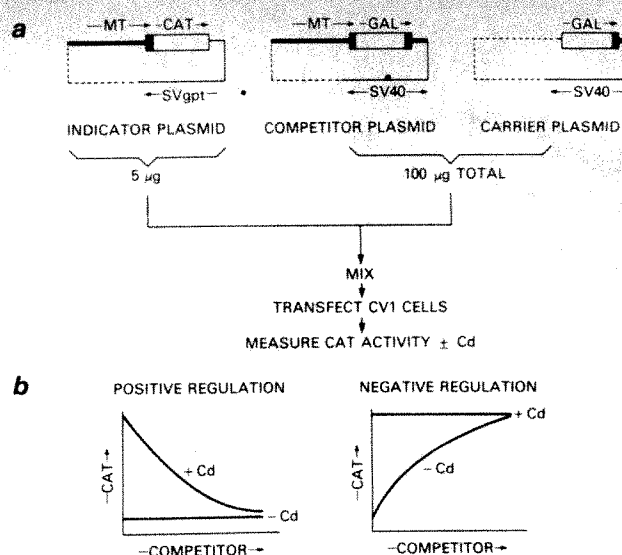


Fig. 1 Design and predicted results of the competition experiment. *a*, Cells were transfected with a mixture of indicator, competitor and carrier plasmids and incubated in the presence or absence of cadmium. Transcription from the *MT* promoter in the indicator plasmid was monitored by CAT enzyme measurements. Solid lines and bars indicate *MT* sequences, hollow bars indicate *E. coli* *CAT* and *gal* sequences, thin lines indicate SV40' sequences, and dotted lines indicate plasmid sequences. *b*, Predicted effects of adding increasing amounts of competitor plasmid on the expression of the *CAT* indicator plasmid. Positively regulated transcription would produce a decrease in the level of cadmium induced expression (left panel), whereas negatively regulated transcription would produce an increase in the level of basal expression (right panel).

Methods: The indicator plasmid, *MT-CAT*, is a derivative of plasmid pSV2-cat (ref. 5) and contains: (1) 2,000 bp 5' flanking sequences and 68 bp 5' untranslated sequences from the mouse *MT-1* gene^{8,9}, inserted into the unique *Hind*III site of the vector through *Eco*RI-*Hind*III and *Bam*HI-*Hind*III adaptors; (2) the coding sequences for *E. coli* *CAT* followed by a fragment of SV40 DNA containing RNA splicing and polyadenylation sites; (3) an SVgpt transcription unit including the SV40 origin of DNA replication and early region promoter followed by the coding sequences for *E. coli* guanine phosphoribosyl transferase and SV40 splicing and polyadenylation sequences¹⁰, inserted at the *Bam*HI site of the vector; (4) *E. coli* plasmid pBR322 sequences including the origin of DNA replication and ampicillin resistance gene. The competitor plasmid, *MT-gal*, has been described previously^{1,8}. The carrier plasmid, previously designated as Δ galI (ref. 1), is identical to the competitor plasmid except that the mouse *MT-1* 5' flanking and 5' untranslated sequences have been deleted and replaced with a *Clal* linker. Plasmids were constructed, characterized and propagated by standard methods¹¹.

factors (Fig. 1b, left panel). Quantitation of the CAT assays showed that the induction ratio for the indicator plasmid fell from 30-fold to 7-fold as the ratio of competitor to indicator plasmid was increased from 0 to 10. Moreover, a plot of the fraction of CAT activity remaining versus the ratio of competitor to competitor-plus-indicator DNA generated close to a straight line with a slope of -1. This is to be expected if the cellular factors interact with the indicator and competitor plasmids with the same affinity. These results were reproducible in multiple experiments ($\sigma = 20-30\%$).

To determine whether the titratable factors are specific for *MT* gene expression, we repeated the competition experiment using an α -globin-*MT* hybrid promoter that is efficiently transcribed but uses globin rather than *MT* upstream regulatory sequences (Fig. 3). This hybrid promoter contains *MT* gene sequences between positions -34 and +68 fused to upstream human α -globin gene sequences between positions -409 and -55 (Fig. 4). The *MT* portion of this construct includes the conserved 'TATA' sequence and the transcription initiation site—both thought to act as sites for interaction with general

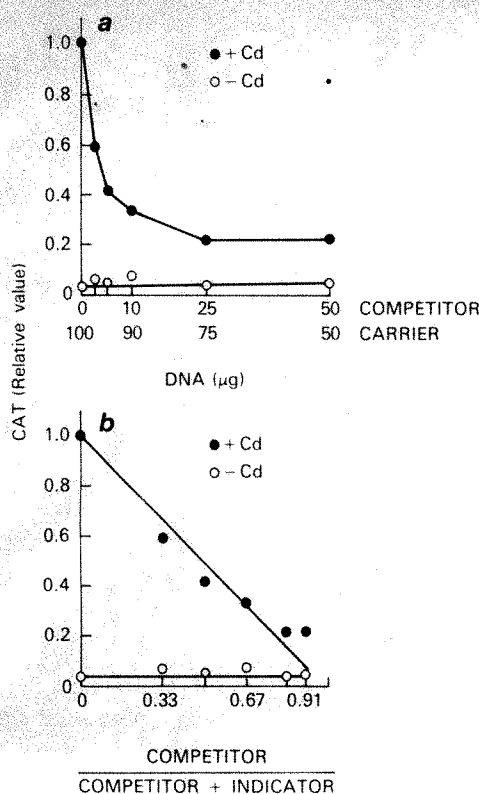


Fig. 2 Transcription is positively regulated. Cells were transfected with 5 μg *MT-CAT* indicator plasmid, 0–50 μg *MT-gal* competitor plasmid and sufficient carrier plasmid to give a total of 105 μg. The cells were incubated in the presence (●) or absence (○) of cadmium and CAT enzyme levels were determined. *a*, Relative CAT values as a function of the concentration of competitor plasmid; *b*, the same data as a function of the ratio of competitor to competitor plus indicator DNA. The relative CAT value is the ratio of CAT activity in the experimental extract to CAT activity in the extract from cells transfected without competitor DNA and induced with cadmium.

Methods: African green monkey kidney cells, line CV1, were seeded into 100-mm culture dishes and grown in Dulbecco's minimum essential medium with 10% fetal calf serum to ~10⁶ cells per plate. Transfection was carried out by calcium phosphate precipitation using a minor modification of previously described methods^{1,5}. Each precipitation mixture contained 10 μg indicator plasmid and 200 μg of competitor plus carrier plasmid. Plasmid DNAs (10–200 μl) were added to 1 ml of 2×HEPES buffered saline (HBS) containing 0.274 M NaCl, 0.01 M KCl, 0.003 M Na₂HPO₄·7H₂O, 0.012 M dextrose, 0.042 M HEPES, pH adjusted to 7.1 with 10 M NaOH. The precipitate was formed by adding 1 ml 0.25 M CaCl₂ dropwise to the DNA/HBS solution with gentle mixing after each drop. The use of 0.003 M phosphate in the HBS solution gave finer precipitates and higher transfection efficiencies than the earlier method. It was also important to purify the plasmid DNA by two centrifugations in CsCl-ethidium bromide gradients. Duplicate plates of cells were incubated with 1 ml of DNA precipitate for 4 h, shocked for 3 min at 37°C with 15% glycerol in 1×HBS, incubated for 12 h in growth medium, then treated or not with 2 μM CdCl₂ for an additional 8 h. Cell extracts were prepared and CAT enzyme activity assayed as described previously⁵.

RNA polymerase II transcription factors—whereas the globin portion contains the upstream *CAT* sequence known to be an essential regulatory element for efficient globin gene transcription in monkey cells⁶. Transcription measurements, either by nuclease S₁ mapping of RNA or galactokinase assays on an α -*MT-gal* fusion gene, showed that this promoter is transcribed efficiently but is not inducible by cadmium (Fig. 4). However, when tested for competition against the *MT-CAT* indicator plasmid, no decrease in CAT activity was observed (Fig. 3b). In the converse of this experiment, we used the *MT-gal* plasmid to compete against an α -*MT-CAT* indicator plasmid and again observed no decrease in CAT activity. We also saw no competition by the *SV40* late and early promoters in an experiment

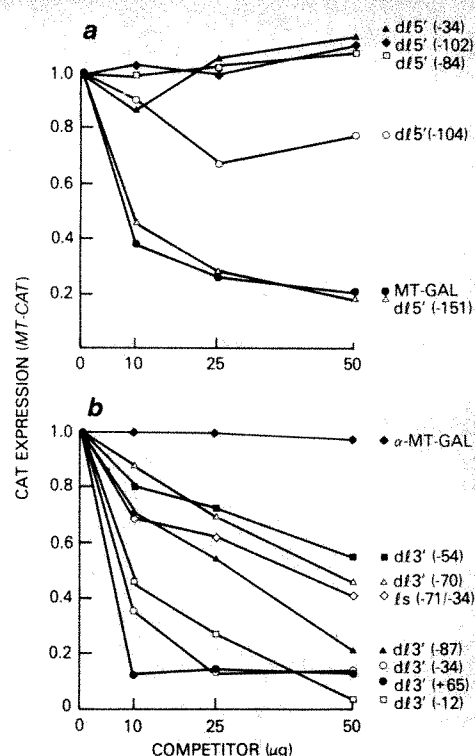


Fig. 3 Competition by deletion mutants and a hybrid promoter. Cells were transfected with 5 μg *MT-CAT* indicator plasmid, 0–50 μg of *MT-gal* or the indicated construct as competitor, and sufficient carrier DNA to give a total of 105 μg. CAT enzyme levels were quantitated and plotted as described in Fig. 2 legend. Only the results for cadmium-induced cells are shown; basal levels were also measured but showed little or no change.

Methods: In the 5' deletion mutants (constructed as described in ref. 1), dl 5'(n), all the *MT* 5' flanking sequences between positions –2,000 and n are replaced by plasmid DNA and the *MT* gene is fused to *gal* at position +68. In the internal deletion, ls(–71/–34), the *MT* sequences between positions –71 and –34 are replaced by a *Cla*I linker and the construct is fused to *gal*. In the 3' deletions, dl 3'(m), the *MT* gene and flanking sequences between positions +2,000 and m are replaced by plasmid DNA. The 3' deletions to +65 and –15 were also fused to *gal* to measure transcription levels in independent experiments. The α -*MT-gal* construct is identical to the standard *MT-gal* competitor plasmid except that the *MT* 5' flanking sequences between positions –34 and –2,000 are replaced by a fragment of the human α -globin gene between positions –55 and –409.

using pBR322-SV40 as competitor and pBR322 as carrier (data not shown). We conclude that the titratable cellular factors are specific for *MT* gene expression and do not represent general RNA polymerase II transcription factors.

To determine the relationship between the DNA sequences responsible for titration of the cellular factors and the transcriptional control sequences of the mouse *MT-I* gene, we performed competition assays on a series of 5', 3' and internal deletion mutants. Figure 3 shows the competition curves and Fig. 4 summarizes the results obtained from previous transcription assays¹ and the present competition experiments. The following results were obtained: (1) A 5' deletion of all the upstream flanking DNA to position –151 competed as well as the starting gene with 2,000 base pairs (bp) of 5' flanking DNA. This mutant was inducible by cadmium and was transcribed ~40% as efficiently as the starting gene. (2) A deletion to position –104 showed very little competition and further deletions to –102 or –84 gave no detectable competition in our assay conditions. (The maximum amount of competitor DNA tested in these experiments was 50 μg, 10-fold greater than the amount of indicator plasmid; higher concentrations of competitor DNA gave irreproducible results.) These mutants were inducible by cadmium but had basal and induced transcription levels only 1–2% of that of the starting gene. A further deletion to position

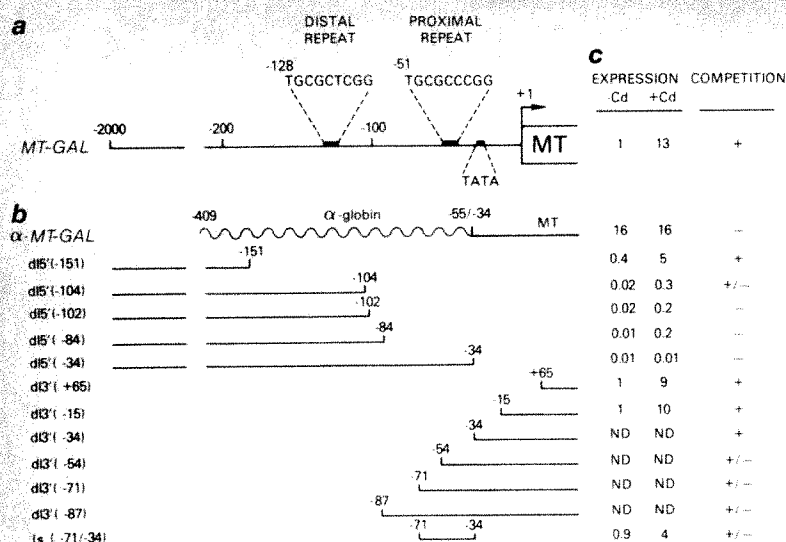


Fig. 4 Relationship between competing DNA sequences and transcriptional control elements. **a**, The structure of the mouse *MT-I* gene 5' flanking region showing the positions and sequences of the repeated nonanucleotide and the conserved TATA box. **b**, The structures of the various constructs used in competition experiments. **c**, Summary of transcription and competition data. The basal and induced transcription levels of these mutants were determined by galactokinase measurements and nuclease *S*₁ mapping of RNA¹; the basal level for the intact promoter is arbitrarily set at 1. The competition curves for these mutants are shown in Fig. 3.

-34 also failed to compete and was not inducible by cadmium. (3) The 3' deletions of the 5' untranslated and flanking sequences to positions +65 or -15 competed as well as the starting gene and were transcribed normally. A deletion to -34, which lacks the conserved TATA box region, also competed efficiently. (4) Further 3' deletions to positions -54, -71 or -87 and an internal deletion between positions -34 and -71 showed partial competition. In each case, two- to eightfold more deletion mutant than wild-type *MT-GAL* DNA was required to obtain a given level of competition. Transcription measurements on the internal deletion mutant showed that it had a normal level of basal expression but a reduced level of expression in the presence of cadmium.

These results demonstrate a strong correlation between the competing DNA sequences and the previously defined distal and proximal heavy metal control regions of the mouse *MT-I* gene. The distal control region, between positions -151 and -78, is essential for both efficient transcription and competition. The proximal region, between positions -78 and -15, is a much weaker transcriptional element and shows no competition in our experimental conditions. The failure of this region to compete is interesting because it gives a normal induction ratio for transcription. Thus it appears that titration of the cellular factor depends on the ability of a DNA sequence to promote efficient transcription in the presence of cadmium, not on the ability to be induced over the basal level. We observed also that the proximal region enhances transcription and competition by factors of two- to eightfold when present in conjunction with the distal region. This may be caused by two regions acting cooperatively or synergistically. For example, weak binding of factors in the proximal region could facilitate stronger binding in the distal region either by protein-protein contacts or by altering the secondary or tertiary structure of the DNA helix.

The distal and proximal control regions contain a duplicated nonanucleotide, present once in the proximal region and once in the distal region, that is conserved in human and rat *MT* genes and that has been implicated in heavy metal regulation^{1,2} (see Fig. 4). These repeats flank a G-rich sequence that may play a role in determining the efficiency of expression. At present we do not know which, if either, of these sequences is responsible for competition. However, it is clear that the nonanucleotide alone is insufficient as 5' deletion mutants retaining one copy of this sequence fail to compete. It is also known that the overall efficiency of *MT-I* gene transcription is affected by the sequences at the TATA box, the cap site and the far upstream region^{1,2}. We show here that these sequences are not responsible for titration of the positively acting cellular components.

We observed specific competition for the cellular regulatory factors only at input DNA concentrations in the range of 5–50 μ g per 10^6 cells, giving intracellular copy numbers of more than 10^6 total plasmids per cell following transfection and replication.

However, because the fraction of the molecules actually available for competition is unknown, we cannot calculate the precise concentration of the regulatory factors. Nevertheless, the high DNA concentrations required, together with the observation that chromosomally amplified *MT* genes are appropriately induced by heavy metals⁷, suggests that there are many active regulatory molecules per cell. We have previously shown that the mouse *MT-I* gene is regulated appropriately when introduced into cells on a replicating viral *SV40* vector⁸, as we observe here good induction ratios in cells transfected with limited quantities (5 μ g per 10^6 cells) of *SV40* plasmid vectors. In these experimental conditions the *MT* control sequences are apparently not present in sufficient quantities to titrate the cellular factors. Thus, in using the competition assay to study the regulation of other genes, it may be necessary to test a wide range of input DNA concentrations to obtain saturation.

The mouse *MT-I* gene shows a substantial level of transcription even when heavy metals are not added to the culture medium. One possible explanation for this is that the cells always contain some intracellular metal and hence some active *MT* transcriptional factors. For example, zinc and copper are inducers of *MT* synthesis which are also required for cell growth and are present in fetal calf serum in substantial concentrations (A. Leone and D.H.H., unpublished results). Alternatively, the *MT* control region may have intrinsic promoter activity even in the absence of active transcriptional factors. The lack of competition for basal transcription at the DNA concentrations tested argues for the second of these models.

The *in vivo* competition assay described here differs in several important aspects from the more traditional transcription assays and may be useful for studying the regulation of other cloned eukaryotic genes. First, the assay allows the formal differentiation between positive and negative transcriptional regulation. Second, because competition can be measured independently of transcription, specific control sequences can be distinguished from general promoter sequences even if they overlap. Finally, the assay can be used to determine whether different genes are regulated by distinct or common cellular factors. We observed no competition between *MT* and *SV40* or *MT* and globin promoters, indicating that they depend on different factors from those described in ref. 4.

The molecular nature of the *MT* regulatory factors is unknown. The present work suggests that at least one class of factors is present in multiple copies and preferentially interacts with the *MT* gene control sequences in metal-stimulated cells.

We thank Mary Jane Walling for technical assistance, Cornelia Gorman for providing *CAT* gene constructs and advice, Cary Queen for comments on the manuscript and Alice Middleton and Beverly Miller for typing. C.S. is supported by a Centennial Fellowship of Le Conseil de recherches médicales du Canada.

received 28 June; accepted 9 November 1984.

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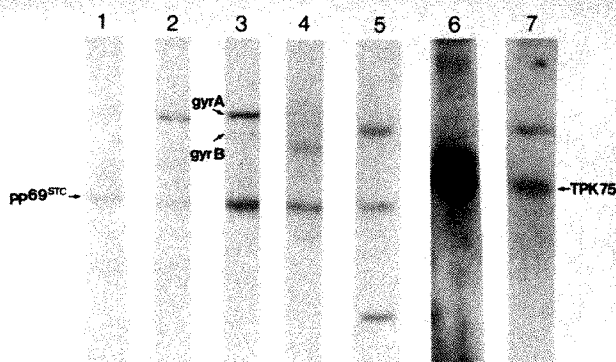


Fig. 1 Autoradiogram of a SDS-polyacrylamide gel showing the *in vitro* phosphorylation of DNA topoisomerases by tyrosine protein kinases. Lane 1, pp60^{src}; 2, pp60^{src} + *E. coli* type I DNA topoisomerase; 3, pp60^{src} + *M. luteus* DNA gyrase; 4, pp60^{src} + calf thymus type I DNA topoisomerase; 5, pp60^{src} + calf thymus type II DNA topoisomerase; 6, TPK75 + calf thymus type I DNA topoisomerase; 7, TPK75 + *E. coli* type I DNA topoisomerase. In each lane, the topoisomerase corresponds to the band of highest molecular weight. The calf thymus DNA topoisomerases used were slightly degraded. Some smaller polypeptides were phosphorylated together with the major ones.

Methods: Each reaction mixture (10-25 μ l) contained 2-5 μ l of either purified pp60^{src} (0.01 unit in 10 μ l, 1 unit being the amount required to transfer 1 pmol of phosphate per min at 30 °C into [Val⁵]angiotensin peptide⁶) from Rous sarcoma virus-transformed rat fibroblast cells, or partially purified TPK75 from rat liver (both preparations were free of cyclic AMP-dependent kinase activity), 10 mM Tris pH 6.8, 3 mM MnCl₂, 0.05% 2-mercaptoethanol, 10 μ Ci [γ -³²P]ATP (3,000 Ci mmol⁻¹) and 1 μ g DNA topoisomerase. After incubation at 30 °C for 30 min, the proteins were precipitated with trichloroacetic acid and analysed by electrophoresis in a 6% SDS-polyacrylamide gel. *E. coli* type I DNA topoisomerase was given by Dr J. C. Wang, and calf thymus type I and type II DNA topoisomerases by Dr L. F. Liu. A sample of calf thymus type I DNA topoisomerase was also purchased from BRL, together with *M. luteus* DNA gyrase.

analysed by thin-layer electrophoresis and chromatography⁹; in all cases phosphorylated residues were found to be tyrosine (data not shown).

We next examined the effect of phosphorylation on topoisomerase activity. After incubation of *E. coli* type I DNA topoisomerase with pp60^{src} and a millimolar amount of ATP, the enzyme was diluted and assayed for its ability to relax supercoiled pUC8 plasmid DNA. Figure 2 (lanes 1, 3) shows that phosphorylation of the enzyme resulted in the loss of > 90% of *E. coli* type I DNA topoisomerase activity; a similar loss of enzyme activity was seen following incubation of calf thymus type I DNA topoisomerase with TPK75 (Fig. 2, compare lanes 7 and 8 with 10 and 11). In separate experiments, we demonstrated that the tyrosine protein kinases had no effect on DNA superhelicity and that incubation of the type I topoisomerases with cyclic AMP-dependent protein kinases from beef heart and rabbit muscle (Sigma) did not result in phosphorylation of the topoisomerases or loss of topoisomerase activity.

We attempted to determine the effect of phosphorylation on the type II topoisomerase activities, but these were much less stable than the type I topoisomerase activities in conditions that were optimal for protein kinase activity. Therefore, even in the control samples without the protein kinase, the type II topoisomerase activities were lost rapidly, making it difficult to evaluate the effect of phosphorylation.

To the best of our knowledge, this represents the first demonstration of modulation of an enzymatic activity other than that of protein kinase via *in vitro* phosphorylation of tyrosine residues of the enzyme. The biological significance of this reaction is presently unknown. Loss of bacterial type I DNA topoisomerase activity *in vivo* results in more highly negatively supercoiled DNA¹⁴. Recent studies on chromatin assembly in *Xenopus* oocytes indicate that in eukaryotes, DNA supercoiling

Virus- and cell-encoded tyrosine protein kinases inactivate DNA topoisomerases *in vitro*

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Tyrosine protein kinase activity is associated with at least eight different retrovirus-encoded *onc* gene products¹ and with cell receptors for epidermal growth factor, platelet-derived growth factor, tumour growth factor and insulin²⁻⁵. Both the *onc* kinases and the growth factor receptors are membrane proteins whose enzymatic activity has been implicated in stimulation of growth. However, the mechanism by which a signal passes from the plasma membrane to the nucleus to initiate growth remains unknown. As DNA topoisomerases catalyse the interconversion of topological isomers of DNA and hence affect DNA replication, transcription and recombination⁶⁻⁸, they may be involved also in stimulation of growth. Several DNA topoisomerases have been shown to form a covalent complex with DNA via a phosphotyrosine linkage^{9,10}. The DNA-protein complex is postulated to be an intermediate in breaking and rejoining of DNA. The aim of the present study was to determine whether tyrosine protein kinases modulate the activity of topoisomerases by phosphorylating the tyrosine residue involved in DNA binding. We report that incubation of *Escherichia coli* and calf thymus type I DNA topoisomerases with the Rous sarcoma virus transforming gene product, pp60^{src}, and TPK75, a tyrosine protein kinase purified from normal rat liver^{11,12}, results in a 10-fold loss of topoisomerase activity.

That an essential tyrosine residue in the active site of topoisomerases acts as a nucleophile is implied by the covalent linkage to DNA via a phosphotyrosine bond^{9,10}. Indeed, chemical modification of the tyrosine residues in *Micrococcus luteus* topoisomerase I and DNA gyrase with tetranitromethane leads to inactivation of the enzyme¹³.

Figure 1 shows the phosphorylation *in vitro* by pp60^{src} of *E. coli* type I DNA topoisomerase (lane 2), *M. luteus* DNA gyrase (lane 3) and calf thymus type I (lane 4) and type II (lane 5) DNA topoisomerases, and the phosphorylation by TPK75 of calf thymus type I (lane 6) and *E. coli* type I (lane 7) DNA topoisomerases. None of the topoisomerase preparations has endogenous protein kinase activity in the condition used here. Subunit A of DNA gyrase (Fig. 1, lane 3), which exhibits the DNA breaking-rejoining activity, was phosphorylated to a much greater extent than subunit B, which possesses the ATPase activity. Examination of the pattern obtained by SDS-gel electrophoresis of the topoisomerases revealed that *E. coli* DNA topoisomerase I and DNA gyrase are homogeneous. The only band visible by staining in the calf thymus topoisomerase I preparation has a relative molecular mass (M_r) of 85,000 (85K) while that of the calf thymus topoisomerase II is 98K. Bands of these relative molecular masses were indeed phosphorylated by the protein kinases. To determine which amino acids in the topoisomerases were phosphorylated, the topoisomerases were eluted from the SDS gel and subjected to acid hydrolysis, then

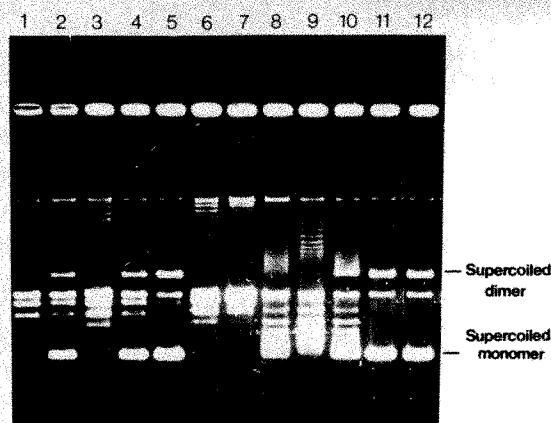


Fig. 2 Agarose gel electrophoresis of plasmid DNA, showing the effect of tyrosine protein kinases on the activity of type I DNA topoisomerases. The following were added to samples of supercoiled DNA: lane 1, 20 ng and lane 2, 2 ng of *E. coli* DNA topoisomerase I incubated without pp60^{src}; lanes 3-5, 200, 20 and 2 ng respectively of *E. coli* DNA topoisomerase I incubated with pp60^{src}; lanes 6-8, 1, 0.1 and 0.01 units respectively of calf thymus DNA topoisomerase I incubated without TPK75; lanes 9-11, 1, 0.1 and 0.01 unit respectively of calf thymus DNA topoisomerase I incubated with TPK75; lane 12, no topoisomerase.

Methods: Topoisomerase and protein kinase were incubated for 60 min in conditions described in Fig. 1 legend, except that 1 mM ATP was used instead of [γ -³²P]ATP. The topoisomerase was then diluted and assayed. The reaction mixture for *E. coli* DNA topoisomerase I contained 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 100 μ g ml⁻¹ gelatin, 6 mM MgCl₂ and 0.5 μ g of supercoiled pUC8 plasmid DNA. The reaction mixture for calf thymus DNA topoisomerase I contained 150 mM NaCl, 20 mM Tris-HCl pH 8, 100 μ g ml⁻¹ gelatin, 1 mM EDTA and 0.5 μ g of supercoiled plasmid DNA. After incubation at 37 °C for 30 min, the reaction was stopped by adding 1% SDS, and the mixture analysed by electrophoresis on a 0.8% agarose gel.

and chromatin assembly are probably coupled *in vivo*¹⁵. The degree of DNA supercoiling in transcriptionally active chromatin is probably controlled by balancing the relaxing activity of DNA topoisomerase I and the supercoiling activity of a 'gyrase' consisting of DNA topoisomerase II coupled with ATP and unknown factor(s). A correlation between the proliferation state of eukaryotic cells and DNA superhelicity has been observed by several investigators¹⁶⁻¹⁸. When the rate of cell growth is high, DNA is more negatively supercoiled, and vice versa¹⁶⁻¹⁸; there is no clear explanation for this alteration of supercoiling. Postsynthetic modification of topoisomerases may represent one possible mechanism of regulating cellular proliferation. The products of retroviral oncogenes, including several membrane-associated tyrosine protein kinases¹⁹, and membrane-associated growth factor receptor tyrosine protein kinases (such as that for epidermal growth factor) share the ability to stimulate growth, possibly through common mechanisms. Such stimulation of growth must involve activation of replicative processes and increased levels of transcription and translation. It will be of interest to determine whether retroviral- or cellular-encoded protein kinases affect topoisomerases either by direct interaction or through another tyrosine protein kinase that shuttles from the plasma membrane to the nucleus, transmitting a growth signal. For example, it is known that the binding of epidermal growth factor to the plasma membrane results in the internalization of a portion of the receptor molecule^{20,21}.

We propose that the receptor kinase might affect growth processes by either migrating to the nucleus and directly phosphorylating a topoisomerase, or by phosphorylating another kinase which in turn passes to the nucleus and phosphorylates a topoisomerase. The same possibilities exist for growth signal transmission by plasma membrane-associated viral tyrosine kinases. However, the activity of such kinases should be independent of an external growth signal. Although there exist no data indicating that viral tyrosine kinases are internalized,

experiments from our laboratory and elsewhere have shown that proteolytic fragments of pp60^{src} contain kinase activity¹⁹. In addition to phosphorylation by tyrosine protein kinases, it has been shown previously that DNA topoisomerases from various sources can be modified *in vitro* by phosphorylation of a serine residue²², or by poly(ADP) ribosylation²³. It remains to be determined which, if any, of these reactions is important *in vivo*.

Received 13 August; accepted 18 October 1984.

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Errata

Unusual abundance of glyceraldehyde 3-phosphate dehydrogenase pseudogenes in vertebrate genomes

M. Piechaczyk, J. M. Blanchard, S. Riaad-el Sabouty, C. Dani, L. Marty & P. Jeanteur*

Nature **312**, 469-471 (1984)

In the issue of 29 November the above letter appeared with an incorrect title.

Modelling the global climate response to orbital forcing and atmospheric carbon dioxide changes

N. G. Pisias & N. J. Shackleton

Nature **310**, 757-759 (1984)

THE legend to Fig. 1 was incorrect and should read:

Fig. 1 Linear variance spectra for *a*, solar insolation at 65° N in June; *b*, the benthic oxygen isotope record from core V19-30; *c*, the simulated ice volume from the simple ice sheet model of Imbrie and Imbrie⁸; and *d*, for the time derivative of the benthic oxygen isotope record from core V19-30. All spectral calculations are from 326 data points spanning the last 340 kyr. Bandwidth shown is for spectral calculations from 120 points of the sample auto-covariance functions. Important periods associated with orbital parameters are labelled.

Physics at the frontier

Abdus Salam

Superforce: The Search for a Grand Unified Theory of Nature.

By Paul Davies.

Simon & Schuster/Heinemann: 1984. Pp.255. \$16.95, £12.95.

Like many compelling images, it may turn out to be a mirage, but for the first time in the history of science we can form a conception of what a complete scientific theory of the world will look like.

Paul Davies in *Superforce*.

THERE is no question but that there has been a transformation, during the past decade, in our understanding of the inanimate physical Universe — a “quantum” jump of the type which from time to time takes place in the history of science. It is this leap in knowledge that Paul Davies sets out to describe in his latest book.

Some eleven years ago, around 1973, the situation was this: we had just begun to believe that all matter in the Universe was made from quarks and leptons (electrons, muons and neutrinos). We believed then that there were four distinct forces controlling all interactions of matter — the universal force of gravity; the force of electromagnetism, acting between electrically charged quarks and leptons; and the two nuclear forces, the strong nuclear force, between quarks, and the weak nuclear force, between left-spinning quarks and leptons. So far as the cosmological structure of the Universe was concerned, observations supported a “standard” model of an expanding Universe; this model stipulated a Universe beginning with a mysterious big bang. We could trace the orderly evolution of the Universe — the formation and emergence of the familiar nuclei, atoms, stars and galaxies — with an epoch starting one-hundredth of a second after time began. But the earlier history was unknown.

The transformation which has taken place since that time lies primarily in the understanding of the nature of the four forces and a comprehension of the earliest history of the Universe. We believe today that the primary agents responsible for the four fundamental forces are the so-called “gauge particles”. The prototype gauge particle is the quantum of light — the photon — which is the “gauge messenger” for the force of electromagnetism. During the past ten years, there has been experimental confirmation of the existence of gauge particles responsible for the strong nuclear force between quarks, and, even more dramatically, the discovery — in 1983 — of the gauge particles of the weak nuclear forces, the so-called Ws and the Zs. Even more important than the unveiling of these objects has been the experimental confirmation of a theory, first proposed in 1967, that the two seemingly distinct forces — electromagnetism and the weak nuclear

force — share a common origin and are indeed aspects of a single unified force, the electroweak force. The clearest manifestation of this unification would need temperatures of the order of 10^{16} K or the laboratory provision of accelerator energies in excess of 100 GeV. Such an accelerator was built at CERN in Geneva in 1982 and the Ws and the Zs were duly created last year, with their theoretically predicted masses, in proton-anti-proton collisions.

So far as the history of the early Universe is concerned, we now believe that there was an epoch — lasting up to 10^{-12} s after time began — when temperatures in excess of 10^{16} K did obtain. During this epoch the Ws and the Zs shared with the photon a state of masslessness. A phase transition took place when temperatures fell below 10^{16} K, concomitant with the expansion of the Universe and its cooling. It was this phase transformation which was responsible for producing the distinction between electromagnetism and the weak nuclear force which we observe today. Before this epoch there was no distinction. With electroweak unification, we have thus pushed back the known life span of the early Universe by ten orders of magnitude.

So much for what is established and non-speculative in the current thinking. With the success of these ideas however, the question arose: are the other two forces, the strong nuclear and the gravitational, also united with the electroweak? Are we dealing in the end with just one force? Twelve years ago, speculation began on this subject. As regards the unification of the strong nuclear force with the electroweak, an attractive version of such a “grand” unification suggested that perhaps another phase transformation did take place some 10^{-31} s after time began. This would extend our knowledge of the early Universe by another 20 orders of magnitude in time.

One hard consequence of such a second unification would be the prediction of the instability of the proton, which in this picture would have a half-life of the order of 10^{38} s. Experimental confirmation of this decay has been sought but not yet conclusively demonstrated. Yet, a phase transition of the type implied in this “grand unification” is not to be abandoned lightly, for this gives the best explanation of the otherwise mysterious predominance of matter over anti-matter as observed in the Universe today. Thus the search for proton decay is still on, and will remain so.

In a finely written book, Paul Davies

takes us through all of these developments. He starts *ab initio*, assuming no prior knowledge of quantum theory or relativity theory, and gives a lucid exposition of these two earlier revolutions in our thinking. He then discusses the unification ideas I have mentioned above, and the relevance of the phase transitions in the early history of the expanding Universe, before moving onto the ideas currently occupying the centre of the theoretical stage which attempt to include the fourth force — gravity — in the scenario. Naturally, these ideas are highly speculative in nature. Among them is the postulate of a new type of symmetry — the so-called supersymmetry — combined with a postulate of extra dimensions of space, in what is known as the Kaluza-Klein framework. The synthesis of these two ideas gives rise to a discussion of the “superforce” of the book’s title, a concept which draws together supermatter (quarks, leptons, gauge particles and their superpartners) into one unified whole with the force of supergravity (described by gravitons and their speculated superpartners, the gravitinos). The theory is not yet in any final shape, but it possesses a beguiling aesthetic attractiveness, particularly when formulated in 11 dimensions.

Davies also gives an account of the associated phase transitions in the earliest history of the Universe, including the recent ideas of its rapid inflationary expansion and the possible origin of the Universe as a “quantum fluctuation” of the vacuum, out of “nothingness”.

This is new, heady stuff, professionally and accurately rendered. However, if I have one criticism of this eminently readable book, it is this. In the final part, one does not get the impression that the author is writing about material which is so highly speculative. The criticism applies also to a comment in the penultimate chapter:

The idea that non-causal, holistic order exists in the universe by no means originated with modern physics. Astrology, for example, is an attempt to discern a cosmic order in which the affairs of human beings are reflected in the organization of the heavens. The psychoanalyst Carl Jung and the quantum physicist Wolfgang Pauli proposed a non-causal connecting principle which they called synchronicity. They compiled evidence for a sort of pervasive order in which apparently independent events occur in conjunction in a meaningful way. Typical of such events are documented instances of extraordinary coincidences, well beyond the expectations of chance.

There are insufficient cautionary warnings to the reader that such thinking — about astrology and “coincidences” — is not the staple of physics. But this apart, unquestionably, Paul Davies has established himself as one of the most felicitous writers on physics at the frontier.

Abdus Salam is Director of the International Centre for Theoretical Physics, Trieste, and a Professor in the Department of Physics at Imperial College, University of London.

Outside eye on the hominid beat

Leonard Krishtalka

Human Evolution: An Illustrated Introduction.

By Roger Lewin.

Blackwell Scientific/W.H. Freeman: 1984. Pp. 104. Hbk \$22.36; pbk £6.80, \$14.95.

PALAEoANTHROPOLOGY is familiar terrain to Roger Lewin. With Richard Leakey, he wrote *Origins* and *People of the Lake*, and he regularly covers the hominid beat, among other subjects, for *Science*. Not surprisingly, *Human Evolution: An Illustrated Introduction* is a testament to Lewin's expertise at science journalism. The writing is deft, the science lucid and the attitude no-nonsense.

All of the main characters in palaeo-anthropology are here — *Aegyptopithecus*, *Ramapithecus*, *Sivapithecus*, the australopithecines and *Homo* — along with the basic plot: the origin and evolution of hominids, bipedalism, large brain size, tool technology and social structures. Lewin ties the fossil record, morphology and, as appropriate, the archaeology of the various taxa to reconstructions of their evolutionary relationships, palaeoecology and cultural adaptations. This core information occupies only 70 or so of the 99 pages. The remainder are devoted to first principles and to the auxiliary disciplines that impinge on hominid palaeontology: highlights of the evolution of life on Earth; primate origins, diversification and disper-

sal; Neogene climates and geology; evolutionary theory; molecular-based phylogenies; taphonomy; ecological theory and more. Lewin also steers a clear and even-handed course through such contentious issues as phyletic gradualism versus punctuated equilibrium, molecular versus palaeontological divergence clocks, the status of *Australopithecus afarensis*, socio-biology and so on.

This is not a personal work. Lewin is the journalist throughout, reporting the consensus and disagreements, and deferring in most instances to the conclusions of the principal investigators in each field. And the presentation is balanced: comprehensive yet concise, not overly technical for the general reader yet meaty enough for most students of human origins. The index is detailed and the selected bibliography adequate.

My one quibble concerns the illustrations. They complement and supplement the text, but curiously are not numbered; nowhere are they tied to the written material. Although most of the figures appear near the appropriate text, the usual in-text references to figure so and so would have been helpful. Also, a few of the figure captions could be more explanatory, especially for those illustrations taken from technical research papers.

Over the past few years palaeoanthropology has matured into a vigorous multidisciplinary science. Lewin's *Human Evolution* is a concise primer on the subject which a wide variety of readers will find rewarding. □

Leonard Krishtalka is Associate Curator in the Section of Vertebrate Fossils, Carnegie Museum of Natural History, Pittsburgh, Pennsylvania.

How green was the Devonian valley?

Keith Allen

Plant Life in the Devonian.

By Patricia G. Gensel and Henry N. Andrews.

Praeger: 1984. Pp. 380. \$29.95, £32.95.

WITHIN palaeobotany, three areas of major and exciting evolutionary change can be distinguished. First, the origin and early evolution of life in the Precambrian; secondly, the rise of vascular land plants in the Upper Silurian and Devonian; and thirdly the origin and evolution of the angiosperms in the Lower Cretaceous.

In 1921 E.A. Newell Arber wrote a small book on Devonian floras. Arber's work supported the growing concept that Devonian land plants included the most primitive of vascular plants, and that this system was one of great evolutionary change, thereby encouraging an increasing number of research workers to study the

Devonian. All the ensuing activity was reflected in the enormous increase in species described, particularly during the past 25 years. But while books have appeared recently on both Precambrian life and angiosperm origins, one or two brief summaries apart there has been nothing on Devonian floras. This new volume by Gensel and Andrews partly satisfies the need for a detailed account of the subject.

Viewing the book as an entity, it seems to me that the authors couldn't decide whether to make it a monograph (though they indicate that it isn't) or a more general work. Much of the book (pp. 36–314) contains good, readable descriptions in partial monographic form of selected species, together with discussions of heterospory and early seeds, and more general comments. An early chapter reviews pre-Devonian plants, particularly the microfloral evidence from the Upper Ordovician and Silurian, and later chapters briefly discuss palynology and Devonian floras.

In a volume which is assembled from sifting through an enormous number of papers there is bound to be some lack of

balance, but the descriptive part of the book is unduly biased towards North American and western European species. Past research was indeed more or less confined to these areas, but Devonian floras have been recorded from the Soviet Union since the 1950s, and there has recently been considerable interest in China. The resulting descriptions are usually brief, but in some instances more could have been added at least for floristic comparisons; for example, no mention is made of the important genus *Arachnoidopteris* occurring in China. Also, in my opinion the authors have not placed enough importance on dispersed spores in the interpretation of Devonian floras. Reference is made to them only in passing in the discussion of heterospory, and in the *in situ* spore studies, yet the sculpture and construction in certain dispersed spores can confidently be used to give further information on age and geographic distributions of floras. Similarly, the section on Devonian floras is too short with the Middle Devonian landscape restricted to less than a page.

There are other points worth questioning. The authors refer to *Cooksonia* as the only undoubted vascular plant found in the Late Silurian, but the evidence is not conclusive that *Cooksonia* is a vascular plant. Even less so is *Steganotheca*, which they include within the vascular family Rhyniaceae, though earlier they mention it within the possible vascular plants. Why, one wonders, aren't *Rhynia gwynne-vaughanii* and *Rhynia major* given separate treatment, rather than being lumped under *Rhynia*, for the "xylem" of *R. major* is clearly very different from that in *R. gwynne-vaughanii*? *Baragwanathia longifolia* is included in the section on Lower and Middle Devonian fossils, where evidence now suggests that the lower *Baragwanathia* horizon is Upper Silurian (to be fair, this latest dating may have been published after the book went to press). It would, too, have been preferable for the descriptions of the rhyniophytes to have preceded rather than followed the trimerophytes; after all, they are less complex, and it seems likely that they gave rise to the trimerophytes. Finally, I am not convinced that a book on Devonian floras should include a detailed account of selected Lower Carboniferous seeds.

In this review I have concentrated on the shortcomings of the book, but, all in all, Gensel and Andrews have done well in compiling such a readable volume. Libraries will doubtless be the main market, though palaeobotanists and taxonomists will be pleased to own a personal copy. For the next edition the authors might consider including more information on palaeoecology, floras and evolutionary trends, which would broaden the potential readership to include third-year undergraduates.

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Essays into history

Christopher Cullen

The Great Inertia: Scientific Stagnation in Traditional China.

By Wen-yuan Qian.

Croom Helm: 1984. Pp. 155. £15.95, \$24.

QIAN is a theoretical physicist who left the Peoples' Republic of China in 1980 after considerable personal suffering during the Cultural Revolution. He is now apparently a permanent exile in the United States, although his wife and daughter are still in China. He has changed disciplines as well as countries: in 1983 he completed an MA in history at Northwestern University, and he is now working for a PhD at the University of Michigan. As Qian himself makes plain, such personal details are not relevant to a judgement on his work.

Qian's book is conceived in the form of an attack on what he takes to be basic errors in the work of Joseph Needham. Most readers of this journal will have at least heard of Needham's huge pioneering study of pre-modern Chinese science, technology and medicine under the title *Science and Civilisation in China*, published by Cambridge University Press since 1954. (Seven volumes are planned in thirty tomes, of which eleven have so far appeared.) Qian mounts his attack in three essays which make up the bulk of his book. The first of these argues that the physical sciences in traditional China remained in a state of "stagnation". The second suggests that social factors such as the high degree of ideological control in China, ancient and modern, were responsible for the failure to develop modern science independently in the past, as well as for the failure to make adequate use of it in the present day. The final essay, written from the point of view of a thorough-going mechanistic reductionism, attacks Needham's view that the Whiteheadian organicism which he believes to be inherent in Chinese thought represents a possible way forward for modern science.

Qian's arguments are presented with clarity of expression and the polemical vigour which comes from deep personal involvement. He is passionately in earnest

when he points to the factors which he believes have held back China in the past and continue to hold her back today, and the whole book is imbued with his sense of relief at being in an environment where he is free to speak his mind. I cannot however commend this short book as a significant contribution to scholarship. Qian's three essays try to settle some very broad issues in the history of science and society in East and West. For such attempts to be worth reading, it is essential for their author to base his generalizations on a solid foundation of first-hand knowledge of the full complexities of the historical evidence. Such an acquaintance usually induces a modest tentativeness in coming to conclusions. Qian however is cocksure and dogmatic throughout: all the important questions are simple and he has found the answers to them. Ironically, what he does know about Chinese science seems to have been drawn in its totality from a study of a few of Needham's published volumes. With disarming frankness (p. 19) Qian tells us that he wrote the second essay of his trio before commencing even this very partial exploration. Qian calls his technique of confident seat-of-the-pants generalization "macrohistory". I am afraid it reminds me of what Jonathan Swift called "the art of being deep learned and shallow read".

The root of Qian's problem is that he seems to believe that to be a modern Chinese trained in modern science is ample qualification for writing on the history of Chinese science and society over the past three thousand years. For comparison, how seriously would we take the work of a modern Greek theoretical physicist who published a rapid review of the cosmology of Aristotle? Qian is simply not going to make any impact on the audience he seeks to influence, which is presumably the small but growing group of scholars who study Chinese science from the original source material. His background may be some excuse for being out of touch, but there is no excuse for those who have encouraged him into such damagingly premature publication. □

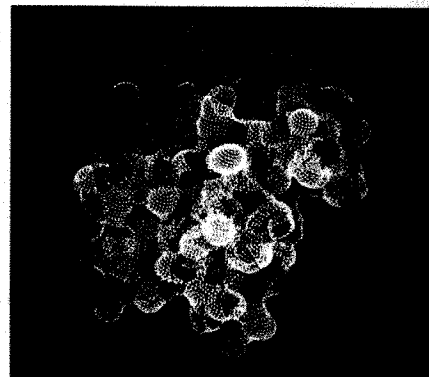
Christopher Cullen is a former Research Fellow of Clare Hall, Cambridge, and has published mainly in the field of ancient Chinese astronomy and mathematics. At present he is Head of Physics at Highgate School, London.



Patterns of agriculture — colour stripping of farmland in Lancaster County, Pennsylvania. The picture is reproduced from *Landprints*, by Walter Sullivan, recently published by Times Books New York (price \$22.50). The book will be reviewed in *Nature* at a later date.

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Questions for the palaeoecologist

Peter D. Moore

Past and Present Vegetation of the Far Northwest of Canada.

By J.C. Ritchie.

University of Toronto Press: 1984.

Pp. 251. \$35, £29.75.

THE most remote corner of Canada, the far Northwest, may seem an unlikely setting for a book about vegetational history. But, as Jim Ritchie points out in his introduction, there are several very good reasons why it should be regarded as a source of interest. In the first place there is its glacial history; rather surprisingly much of the area has escaped major glaciation, certainly for the past half-million years, so we might well expect long, undisturbed profiles of stratified sediments to have accumulated there. Then there is the fact that the region forms the eastern edge of Beringia — the last terrestrial point of contact between the Old World and the New across what are now the Bering Straits. Add to this the wide range of climatic regimes and vegetation types, and the Northwest can be seen to be full of promise for the palaeoecologist.

Ritchie begins by setting the physical and biological scene for the palaeovegetation studies. His emphasis is upon the contrasting environments of the Northwest which result from the gradient between the continental-subarctic climate with early springs in the south, and the arctic-coastal climate with late springs in the north. But the data presented here are not always easy to appreciate simply because there is no map of the locations named. Lack of a map also makes it difficult to interpret some of the intriguing periglacial geomorphology of the region. Take pingos, for example. How does their distribution relate to the limits of glaciation? Are they located only on unglaciated terrain?

A very helpful map of the major vegetation zones is supplied covering not only the Northwest, but also Alaska and eastern Siberia, and is supplemented by outline descriptions of the vegetation types of the area. Detailed lists and phytosociological analyses are given in appendices.

But the main part of the book is given over to the history of vegetation and reconstruction of palaeoenvironments, starting from Tertiary times but concentrating upon the Quaternary. Much of the Tertiary data, which must by now be available as a result of oil exploration, is still hidden from the public gaze and scientific scrutiny.

In view of the unglaciated nature of much of the Northwest terrain, one might hope for long cores of sediment recording Quaternary vegetation history; sadly, this is not the case. Ancient sediments, perhaps dating back about 700,000 years, have been

found in river-bank exposures but no continuous core has yet been obtained.

The last 13,000 years are very well documented from many sites, largely as a result of Ritchie's own work, and the results not only provide a picture of the changing vegetation of the Northwest, but also illustrate many of the problems currently facing palaeoecologists. How do we interpret pollen assemblages for which there is apparently no modern analogue? What are the relative merits and limitations of percentage and influx diagrams? What interpretive allowances must be made for the size of sampling site?

The mass of data and the wealth of

problems described are finally pulled together in a general reconstruction of environmental history. This particular approach results in the combined advantages of a full and detailed picture of the changing vegetation of this interesting area, together with the salutary lessons to be gained from the practical problems encountered in the process. The latter point will make the book valuable even to palaeoecologists whose research interests are far removed from the remote Northwest.

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Fresh fusion

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I.H. Hutchinson

Introduction to Plasma Physics and Controlled Fusion, 2nd Edn. Vol. 1 Plasma Physics.

By Francis F. Chen.

Plenum: 1984. Pp. 421. \$24.50, £23.28.

Fusion: An Introduction to the Physics and Technology of Magnetic Confinement Fusion.

By Weston M. Stacey, Jr.

Wiley: 1984. Pp. 260. \$39.95, £46.20.

WHEN an author publishes a second book covering a specialized scientific topic, as is the case for both books reviewed here, the immediate question that springs to mind is "why?". What is the relationship of the new book to his earlier one? In the case of Francis F. Chen's *Introduction to Plasma Physics and Controlled Fusion* the answer is straightforward: in this second edition of a work originally published in 1974, he plans an expansion of the fusion section into a complete volume, the plasma physics now also taking an entire book. We shall await the appearance of that second volume with interest; meanwhile the present book, apart from some corrections, valuable additions to the chapter on nonlinear effects, and a change to SI units, deviates very little from its predecessor. This is perfectly justifiable since Chen's book has become widely accepted as an excellent introductory text in plasma physics. Its emphasis on intuitive understanding and the copious sketches and diagrams help to make it truly accessible to undergraduates in both physics and engineering.

The author's objective of including where possible all algebraic steps in the development is achieved with surprisingly few tedious sections; however, sometimes this principle leads him to omit the derivation entirely when an outline would have been helpful. It is hard for the student to rectify this failing using other sources because the references are limited mostly to papers whose results are included in figures in the book. Such minor points apart, this

is a deeply satisfying book, full of insight and with a very happy choice of material for a first course in plasma physics. The serious student will want to go further, but this is a good place to start.

The question "why" is more difficult to answer for Weston M. Stacey's *Fusion*. Neither the subtitle, *An Introduction to the Physics and Technology of Magnetic Confinement Fusion*, nor the preface are of much help. The first half of the book consists of an overview of the plasma physics of magnetic confinement. Most of the material, including 18 of the figures, abstracted from Stacey's *Fusion Plasma Analysis* which appeared only three years ago. Whatever the merits of the earlier book, abbreviating the material for the present work has resulted in a distinctly light-weight treatment. Such an introduction is perhaps appropriate for a non-specialist student; certainly the text is easy to read; but unfortunately it is hard to escape the feeling that simplification has led to superficiality.

The latter half of the book consists of an introduction to the engineering and technology involved in fusion reactor design. Of great merit here is the wealth of physical data included in the form of tables and graphs, which lend a valuable tone of solidity to the engineering issues. Sections on nuclear activation and tritium breeding and handling are particularly welcome. But again the brevity of the treatment and the frequent lack of a proper explanation of the quantities under discussion leaves the reader uncertain of the rigour of any conclusions.

For someone wanting to become broadly acquainted with the various aspects of fusion research, Stacey's book offers a readable introduction. But it is not one which lays a particularly firm technical foundation for detailed research in the field.

I.H. Hutchinson is an Associate Professor in the Department of Nuclear Engineering at the Massachusetts Institute of Technology.

● Also recently published by Wiley is *Fusion Energy*, by Robert A. Gross, a graduate-level introduction to the principles behind fusion science and technology. Price is \$41.95, £42.